

A peer-reviewed version of this preprint was published in PeerJ on 4 May 2018.

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Wangensteen OS, Palacín C, Guardiola M, Turon X. 2018. DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. PeerJ 6:e4705 <https://doi.org/10.7717/peerj.4705>

Metabarcoding littoral hard-bottom communities: unexpected diversity and database gaps revealed by two molecular markers

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We developed a metabarcoding method for biodiversity characterization of structurally complex natural marine hard-bottom communities. Novel primer sets for two different molecular markers: the “Leray fragment” of mitochondrial cytochrome c oxidase, COI, and the V7 region of ribosomal RNA 18S were used to analyse eight different marine shallow benthic communities from two National Parks in Spain (one in the Atlantic Ocean and another in the Mediterranean Sea). Samples were sieved into three size fractions from where DNA was extracted separately. Bayesian clustering was used for delimiting molecular operational taxonomic units (MOTUs) and custom reference databases were constructed for taxonomic assignment. We found unexpectedly high values for MOTU richness, suggesting that these communities host a large amount of yet undescribed eukaryotic biodiversity. Significant gaps are still found in sequence reference databases, which currently prevent the complete taxonomic assignation of the detected sequences. Nevertheless, over 90% (in abundance) of the sequenced reads could be successfully assigned to phylum or lower taxonomical level. This identification rate might be significantly improved in the future, as reference databases are updated. Our results show that marine metabarcoding, currently applied mostly to plankton or sediments, can be adapted to structurally complex hard bottom samples, and emerges as a robust, fast, objective and affordable method for comprehensively characterizing the diversity of marine benthic communities dominated by macroscopic seaweeds and colonial or modular sessile metazoans, allowing for standardized biomonitoring of these ecologically important communities. The new universal primers for COI can potentially be used for biodiversity assessment with high taxonomic resolution in a wide array of marine, terrestrial or freshwater eukaryotic communities.

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16 **Keywords:** biodiversity assessment, cytochrome *c* oxidase I, eukaryotic communities, marine
17 benthic ecosystems, metabarcoding pipelines, ribosomal RNA 18S

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20 **Running title:** Metabarcoding hard-bottom communities

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26

27 **Abstract**

28

29 We developed a metabarcoding method for biodiversity characterization of structurally complex
30 natural marine hard-bottom communities. Novel primer sets for two different molecular markers:
31 the “Leray fragment” of mitochondrial cytochrome *c* oxidase, COI, and the V7 region of
32 ribosomal RNA 18S were used to analyse eight different marine shallow benthic communities
33 from two National Parks in Spain (one in the Atlantic Ocean and another in the Mediterranean
34 Sea). Samples were sieved into three size fractions from where DNA was extracted separately.
35 Bayesian clustering was used for delimiting molecular operational taxonomic units (MOTUs)
36 and custom reference databases were constructed for taxonomic assignment. We found
37 unexpectedly high values for MOTU richness, suggesting that these communities host a large
38 amount of yet undescribed eukaryotic biodiversity. Significant gaps are still found in sequence
39 reference databases, which currently prevent the complete taxonomic assignment of the detected
40 sequences. Nevertheless, over 90% (in abundance) of the sequenced reads could be successfully
41 assigned to phylum or lower taxonomical level. This identification rate might be significantly
42 improved in the future, as reference databases are updated. Our results show that marine
43 metabarcoding, currently applied mostly to plankton or sediments, can be adapted to structurally
44 complex hard bottom samples, and emerges as a robust, fast, objective and affordable method for
45 comprehensively characterizing the diversity of marine benthic communities dominated by
46 macroscopic seaweeds and colonial or modular sessile metazoans, allowing for standardized
47 biomonitoring of these ecologically important communities. The new universal primers for COI

48 can potentially be used for biodiversity assessment with high taxonomic resolution in a wide
49 array of marine, terrestrial or freshwater eukaryotic communities.

50 Introduction

51
52 Reliable methods for accurately and objectively assessing the biodiversity of marine
53 environments are needed for a good understanding of these key ecosystems (Costello et al. 2010)
54 in order to establish biodiversity baselines and monitor long-term biodiversity changes
55 (Knowlton & Jackson 2008). Among marine ecosystems, shallow benthic hard-bottom
56 communities are frequently considered to support the highest values of diversity, being arguably
57 the most diverse ecosystems in the biosphere (Reaka-Kudla 1997; Agardy et al. 2005). Their
58 proximity to humans place them among the best studied and most heavily impacted of all marine
59 biomes. They are also the most influential for human ecology and economy. However, marine
60 ecologists still lack robust, standardized tools for comprehensively surveying these communities.

61 An exhaustive analysis of these biomes by traditional morphological methods is impracticable
62 due to their high complexity, the colonial or modular morphology of many groups and the
63 abundance of tiny epibiotic forms (Mikkelsen & Cracraft 2001; Wangensteen & Turon 2017). In
64 most instances, morphological surveys are limited to macro-organisms, and are often focused on
65 a few taxonomic groups, strongly conditioned by the availability of taxonomic expertise. The
66 taxonomic impediment (Wheeler et al. 2004) and the occurrence of cryptic species complexes
67 (Knowlton 1993) further hinder the practicability of morphology-based methods.

68 In the last few years, the development of metabarcoding techniques, whereby thousands of
69 species present in a given environmental sample can be detected by high-throughput sequencing
70 and identified using molecular databases (Hajibabaei et al. 2011; Taberlet et al. 2012), has
71 revolutionized biodiversity assessment. Metabarcoding approaches have been successfully used
72 to characterize marine communities in relatively homogeneous substrates such as seawater (e.g.
73 de Vargas et al. 2015; Chain et al. 2016) or marine sediments (e.g. Chariton et al. 2010; Fonseca
74 et al. 2014; Pawlowski et al. 2014; Guardiola et al. 2015; Lejzerowicz et al. 2015) containing
75 mostly small-sized organisms. Leray & Knowlton (2015) introduced methods for analysing the
76 community DNA extracted from organisms collected in autonomous reef monitoring structures
77 (ARMS) using COI metabarcoding. These artificial collectors have been used to analyse other
78 genetic markers such as 18S (Pearman et al. 2016). However, metabarcoding methods have been
79 scarcely used to characterize complex communities dwelling on marine natural hard-bottom
80 substrates. These environments pose new challenges related to sample treatment (given the

81 orders-of-magnitude variation in organisms' sizes) and to the lack of reliable nearly-universal
82 primer sets, capable of amplifying the wide array of taxonomic groups inhabiting these
83 communities.

84 In the present work, we introduce a metabarcoding protocol for characterizing complex
85 communities inhabiting natural marine hard substrates. The suitability and robustness of our
86 methods are assessed by comparing the results from two independent universal eukaryotic
87 molecular markers: a fragment of the multiple-copy nuclear gene for the small subunit of the
88 ribosomal RNA (18S) and a fragment of the cytochrome *c* oxidase subunit I mitochondrial gene
89 (COI). A multigene metabarcoding approach has been advocated to overcome limitations
90 inherent to single marker studies (Drummond et al. 2015, Coward et al. 2015). The enhanced
91 taxonomic resolution of COI (Tang et al. 2012) is partly counteracted by the lack of universality
92 of most primer sets used for COI amplification, which may fail to amplify some eukaryotic taxa
93 (Deagle et al. 2014). To overcome this problem, we introduce a new primer set, featuring a high
94 ratio of degenerate positions, designed for enhancing universality in the amplification of the
95 "Leray fragment" (Leray et al. 2013) of COI in most eukaryotic groups.

96 We tested this methodology by studying eukaryotic biodiversity patterns on eight different
97 shallow benthic communities (fig. 1) sampled from two marine national parks in Spain (one in
98 Western Mediterranean, and another in Northeastern Atlantic). Size fractionation has been
99 proposed as a necessary step in metabarcoding when organisms on a sample have unequal
100 biomass (Elbrecht et al. 2017). This procedure allowed recovery of 30% more taxa of freshwater
101 invertebrates than unsorted samples (Elbrecht et al. 2017). Size fractionation has been used in the
102 marine environment for metabarcoding macrobenthos in sediment samples (e.g., Aylagas et al.
103 2016), mobile organisms in settlement plates (e.g., Leray & Knowlton 2015, Ransome et al.
104 2017), or zooplankton (e.g., Liu et al. 2017), but it has never been applied to samples with
105 organisms spanning several orders of magnitude in size such as hard-bottom communities (from
106 macrophytes to microbes). We sieved each sample into three size fractions, which corresponded
107 to the distinction between mega-, macro- and meiobenthos (Rex & Etter 2010), a separation with
108 important correlates in terms of structure and function of benthic communities (e.g., Warwick &
109 Joint 1987, Galerón et al. 2000, Rex et al. 2006).

110 Total community DNA was extracted separately for each fraction, and each extract was then
111 metabarcoded in parallel runs using 18S and COI markers. We also analyzed unsieved sediment

112 samples from a tidal lagoon for comparative purposes. Our main objective was to develop and
113 apply a method for characterizing complex marine hard-substrate communities using DNA
114 metabarcoding. To this end, we (1) tested the effects of size-fractionation in the detection of
115 marine taxa spanning widely different sizes, (2) assayed a modified primer set for COI, (3)
116 generated new reference databases, (4) compared the relative performance of 18S and COI in
117 terms of taxonomic accuracy and biodiversity patterns obtained, and (5) generated baseline
118 information for biodiversity assessment and biomonitoring of benthic communities in Marine
119 Protected Areas.

120

121 **Materials and Methods**

122

123 Sampling

124 Samples were taken by scuba diving from different shallow hard-bottom communities inside two
125 national parks in Spain: Cíes Islands (Atlantic Islands National Park, Galicia, Northeastern
126 Atlantic, 42.22°N, 8.90°W) and Cabrera Archipelago National Park (Balearic Islands, Western
127 Mediterranean, 39.13°N, 2.96°E). A map of sampling locations is shown in fig. S1. The rationale
128 for the choice of the communities was to have the most representative habitats along a depth
129 gradient of the rocky littoral of these national parks for the purpose of obtaining baseline
130 inventories for future monitoring and management efforts. Atlantic communities were sampled
131 in May 2014, from two different communities of photophilous algae (3-5 m deep), one
132 dominated by *Cystoseira nodicaulis* (a) and another dominated by *Cystoseira tamariscifolia* (b),
133 a sciaphilous community dominated by *Saccorhiza polyschides* (16 m deep) (c) and detritic
134 rhodolith beds (maërl bottoms) (ca. 20 m deep) (d). Mediterranean communities were sampled in
135 September 2014 from a photophilous algal assemblage dominated by *Lophocladia lallemandii*
136 (e), a photophilous community with an heterogeneous algal composition (f) (both from 5-10 m
137 deep), a sciaphilous precoralligenous community (30 m deep) (g), and detritic rhodolith beds (ca.
138 50 m deep) (h). Although rhodolith beds are not strictly rocky communities, they are included in
139 this work because they share with them the three-dimensional complexity and much of the
140 biodiversity present, as we sampled communities just adjacent to rocky slopes. These detritic
141 communities were sampled (three replicates each) by using a cylindrical PVC corer with a
142 diameter of 30 cm and a height of 5 cm. All other hard-bottom communities (three replicates
143 each) were sampled by carefully scraping a 25x25 cm quadrat with chisel and hammer. All

144 samples were placed underwater inside polyethylene bags. Water was eliminated through a 63
145 μm mesh sieve shortly after sampling, being then replaced by 96% ethanol. The material retained
146 in the filter was washed back to the sample bag with ethanol. Samples were stored at $-20\text{ }^{\circ}\text{C}$ upon
147 arrival to the laboratory, until further processing. For comparative analyses, three additional
148 samples from soft-bottom sandy sediments were taken using a corer (3.5 cm in diameter) during
149 the low tide from a tidal lagoon in Cíes Islands (Lago dos Nenos, 42.223°N , 8.905°W) and
150 preserved whole in 96% ethanol.

151

152 Sample pre-treatment and DNA extraction

153 The samples were separated into three size fractions (A: $> 10\text{ mm}$; B: $1 - 10\text{ mm}$; C: $63\text{ }\mu\text{m} - 1$
154 mm) using a column of stainless steel sieves (www.cisa.net), washing thoroughly under high-
155 pressure freshwater. All separated fractions were then recovered in 96% ethanol, homogenized
156 using a 600 W hand blender and stored at $-20\text{ }^{\circ}\text{C}$ until DNA extraction. All equipment was
157 thoroughly washed and cleaned with diluted sodium hypochlorite between successive samples.
158 The three sediment samples from the lagoon were processed directly without any sieving, and
159 manually homogenized. For total DNA extraction, 10 g of each homogenized sample were
160 purified using PowerMax Soil DNA Isolation Kit (www.mobio.com). DNA concentration of
161 purified extracts was assessed in a Qubit fluorometer (www.lifetechnologies.com) and, if
162 needed, concentrated in a Speedvac system (www.thermoscientific.com) until DNA
163 concentration of $> 5\text{ ng}/\mu\text{l}$ was achieved.

164

165 Reproducibility and negative controls

166 One of the homogenized samples (from the Atlantic community dominated by *Cystoseira*
167 *tamariscifolia*) was extracted in triplicate and amplified independently, in order to check the
168 reproducibility of the DNA extraction procedure. One of these extractions was then amplified
169 using three PCR reactions with different sample tags, in order to check the reproducibility of the
170 PCR amplification and the possible bias introduced by mismatches due to sample tags
171 (O'Donnell et al. 2016). The variability of these samples (due to random errors during the PCR
172 in the latter case, and due to the addition of random errors during the PCR plus the variability in
173 the DNA extraction procedure in the former case) was also compared with the natural ecological

174 variability assessed by the three different replicates obtained from the same community.
175 Two different kinds of negative controls were used during the process. A standard PCR-blank
176 was amplified using the elution buffer of the DNA isolation kit as a sample. A negative control
177 for the pre-treatment separation protocol was done by using a sand sample charred in a muffle
178 furnace at 400 °C for 24 h to remove all traces of DNA. This muffled sand was sieved and
179 extracted using the same procedure used for the samples. 2 PCR-blanks and 2 negative controls
180 were run alongside the samples in the same sequencing plates.

181

182 DNA amplification and library preparation

183 Two different metabarcoding markers were amplified: 18S and COI. For the V7 region of 18S
184 rRNA, the recently developed 18S_allshorts primers were used: forward: 5'-
185 TTTGTCTGSTTAATTSCG-3' and reverse: 5'-TCACAGACCTGTTATTGC-3 (Guardiola et al.
186 2015). These primers show a marked universality across eukaryotic groups (see *in silico* analysis
187 and primer logos in Guardiola et al. 2015). To these primers, 8-base sample-specific tags were
188 attached (the same tag at both ends in order to detect intersample chimeric sequences). The PCR
189 conditions followed Guardiola et al. (2015), using a standardized amount of sample (10 ng of
190 purified DNA per sample).

191 For the 5' region of COI, we used a new highly degenerated primer set (henceforth Leray-XT),
192 which includes the reverse primer jgHCO2198 5'-TAIACYTCIGGRTGICCRAARAAAYCA-3'
193 (Geller et al. 2013) and introduces a novel forward primer mlCOIintF-XT 5'-
194 GGWACWRGWTGRACWITITAYCCYCC-3', modified from the mlCOIintF primer (Leray et
195 al. 2013) by incorporating two more wobble bases and two inosine nucleotides in the most
196 degenerate positions, for increased universality across eukaryotic groups. This was done after
197 manually checking the original primer against representative sequences of the main eukaryotic
198 groups obtained from the Genbank database. Sample tags were attached to both ends of the
199 primers as before. Amplification of COI used AmpliTaq Gold DNA polymerase, with 1 µl of
200 each 5 µM forward and reverse 8-base tagged primers, 3 µg of bovine serum albumin and 10 ng
201 of purified DNA in a total volume of 20 µl per sample. The PCR profile included a denaturing
202 step of 10 min at 95 °C, 35 cycles of 94 °C 1 min, 45 °C 1 min and 72 °C 1 min and a final
203 extension of 5 min at 72 °C.

204 After PCR, quality of amplifications was assessed by electrophoresis in agarose gel. All PCR
205 products were purified using Minelute PCR purification columns (www.qiagen.com) and pooled
206 by marker (83 samples per marker, including blanks and replicates used for the reproducibility
207 study). Two Illumina libraries were built from the DNA pools using the Metafast protocol at
208 Fasteris SA (Plan-les-Ouates, Switzerland, www.fasteris.com). This protocol incorporates
209 Illumina adapters using a ligation procedure without any further PCR step, thus minimising
210 biases. Each library was sequenced independently in an Illumina MiSeq platform using v3
211 chemistry (2x150 bp paired-end run for 18S and 2x300 bp paired-end run for COI).

212

213 *In silico* evaluation of the new COI primer set

214 We tested *in silico* the coverage of the new primer set for COI and compared it with the original
215 Leray set (Leray et al. 2013). This comparison was done for all metazoan phyla and for the rest
216 of eukaryotic groups for which enough sequence information was available. A set of full
217 sequences for COI was downloaded from Genbank and the ability of each primer set to amplify
218 the different species was assessed using *ecopcr* and the *ecotaxstat* function (Ficetola et al. 2010),
219 allowing 3 mismatches per primer. This approach cannot be used directly with partial COI
220 sequences (corresponding to the standard barcoding region), since these barcodes lack the
221 reverse primer binding sequence. Thus, for those eukaryotic groups where not enough complete
222 COI sequences were available (Dinoflagellata, Rhodophyta and Stramenopiles), we ran *ecopcr*
223 and *ecotaxstat* against sets of COI barcode sequences with an artificial jgHCO2198-matching
224 sequence attached to the 3' end. This allowed us to test the coverages of the internal forward
225 primers and to compare the performance of the new Leray-XT primer set to that of the original
226 Leray primers, since both sets are sharing the reverse primer. Primer logos (Crooks et al. 2004)
227 were also obtained to summarize conservation of primer sequences across all eukaryotic groups.

228

229 Metabarcoding pipeline

230 We based our metabarcoding pipeline on the OBITools software suite (Boyer et al. 2016). The
231 length of the raw reads was trimmed to a median Phred quality score higher than 30, after which
232 paired-reads were assembled using *illumina-paired-end*. The reads with alignment quality scores
233 higher than 40 were demultiplexed using *ngsfilter*. A length filter (*obigrep*) was applied to the

234 assigned reads (75 – 180 bp for 18S and 300 – 320 bp for COI). The reads were then dereplicated
235 (using `obiuniq`) and chimeric sequences were detected and removed using the `uchime_denovo`
236 algorithm implemented in `vsearch` (<http://github.com/torognes/vsearch>). The MOTUs were then
237 delimited using the Bayesian clustering algorithm implemented in CROP (Hao et al. 2011). This
238 algorithm results in variable thresholds for delimiting MOTUs across different branches of the
239 taxonomic tree, following the natural organization of the clusters in multidimensional sequence
240 space. The following parameter sets were used: $l=0.3$, $u=0.5$ for 18S (Guardiola et al. 2016) and
241 $l=1.5$, $u=2.5$ for COI. These values were chosen to avoid overclustering of several species into
242 single MOTUs (Wangenstein & Turon 2017).

243 The taxonomic assignment of the representative sequences for each MOTU was performed using
244 `ecotag` (Boyer et al. 2016), which uses a local reference database and a phylogenetic tree-based
245 approach (using the NCBI taxonomy) for assigning sequences without a perfect match. `Ecotag`
246 searches the best hit in the reference database and builds a set of sequences in the database which
247 are at least as similar to the best hit as the query sequence is. Then, the MOTU is assigned to the
248 most recent common ancestor to all these sequences in the NCBI taxonomy tree. With this
249 procedure, the assigned taxonomic rank varies depending on the similarity of the query
250 sequences and the density of the reference database. For 18S, we used the `db_18S_r117`
251 reference database (Guardiola et al. 2015), obtained by *in silico* ecoPCR (Ficetola et al. 2010)
252 with the `18S_allshorts` primer set against the release 117 of the EMBL nucleotide database. This
253 database includes 26,125 reference sequences from all major eukaryotic groups. For COI, we
254 developed a mixed reference database by joining sequences obtained from two sources: *in silico*
255 ecoPCR against the release 117 of the EMBL nucleotide database and a second set of sequences
256 obtained from the Barcode of Life Datasystems (Ratnasingham & Hebert 2007) using a custom
257 R script to select the Leray fragment. This newly generated database (`db_COI_MBPK`) included
258 188,929 reference sequences (March 2016) from a wide taxonomic range. Both reference
259 databases are publicly available from <http://github.com/metabarpark/Reference-databases>.

260 After taxonomic assignment, the final refining of the datasets included taxonomic clustering of
261 MOTUs assigned to the same species, minimal abundance filtering (unassigned MOTUs with
262 less than 10 reads and assigned MOTUs with less than 5 reads were deleted), blank correction
263 and abundance renormalization to remove spurious false positive results due to random tag
264 switching (Wangenstein & Turon 2017). Since we were interested only in eukaryotic diversity,

265 all MOTUs assigned to prokaryotes or to the root of the Tree of Life were removed from the
266 analyses. Samples having less than 10,000 reads in the final datasets, after all filtering
267 procedures, were considered as failed and deleted from the analyses. The pipelines used for both
268 metabarcoding markers are summarized in supplementary material, table S1.

269

270 Statistical analyses

271 All analyses were performed in R v 3.3.0 (<https://www.R-project.org/>). Package vegan (Oksanen
272 et al. 2016) was used for rarefaction analyses (function `rrarefy`), calculations of Bray-Curtis
273 dissimilarity matrices (function `vegdist`), comparison of these matrices (function `mantel`), and
274 group representation in nMDS diagrams (functions `isoMDS`, `ordiellipse` and `ordispider`).
275 Rarefaction analyses for α -diversity were carried out using a rarefaction size of 10,000 reads and
276 500 bootstrap replicates per sample. Package `vioplot` (Adler 2005) was used for plotting the
277 results as violin plots and package `dunn.test` (Dinno 2017) was used to test differences in α -
278 diversity between fractions. All calculations of Bray-Curtis dissimilarities were performed using
279 fourth root-transformed abundance values of relative frequencies (normalized by dividing read
280 abundances of each MOTU by the total reads for each sample). For assessing the reproducibility
281 of the extraction and PCR procedures, weighted UniFrac distances between technical replicates
282 were calculated using package `phyloseq` (McMurdie & Holmes 2013) and compared with the
283 same distances obtained from ecological replicates. This method takes into account not only
284 differences in abundances of the occurring MOTUs for calculating dissimilarities between
285 samples, but also the phylogenetic distance between these MOTUs. For assessing the effect of
286 size fractionation on the detectability of organisms with different sizes, we classified the MOTUs
287 into the following ecological size-categories: (1) macroscopic seaweeds, (2) modular metazoa,
288 (3) macrofaunal unitary metazoa, (4) meiofaunal metazoa, (5) microorganisms and (6)
289 unassigned. The percentages of reads obtained for each category in the three size fractions were
290 compared for each hard-bottom community and to the sediment samples from the tidal lagoon.

291

292 **Results**

293

294 *In silico evaluation of the new COI primer set*

295 The taxonomic coverage at the species level of the new Leray-XT primers and the original Leray

296 set is shown in fig. 2 for different groups of eukaryotic and metazoan phyla. The coverage of the
297 new Leray-XT primers was enhanced for most eukaryotic groups compared to the original Leray
298 set, and reached values higher than 96% for all major groups analyzed, except for Fungi,
299 Viridiplantae and Ciliophora. Values of 96% or higher were also reached for all major metazoan
300 phyla, except for Cnidaria, Platyhelminthes and Porifera. Even for these three phyla, there was a
301 substantial coverage enhancement respect to the original Leray set (59% to 89% for Cnidaria,
302 35% to 82% for Platyhelminthes and 67% to 89% for Porifera). The combined coverage for all
303 Metazoa with the Leray-XT primers was 96.1%, compared with 86.0% for the original Leray set.
304 The primer logos for a combined set of 149,908 COI sequences from 38 different phyla of all
305 eukaryotic lineages are shown in fig. S2.

306

307 *Taxonomic summary of the new reference databases*

308 The number of sequences from different taxonomic groups included in the reference databases
309 used for our analyses is summarized in table S2. Although the total number of different reference
310 sequences for COI is one order of magnitude higher than for 18S, some important taxonomic
311 groups are remarkably absent from the COI reference database, such as Choanozoa, Foraminifera
312 or several fungal phyla, while others are scarcely represented, such as Cercozoa, Excavata or
313 Cryptophyta. Among groups with macro-organisms, the low representation of Viridiplantae in
314 the COI database is noteworthy, while Chordata are poorly represented in the 18S database
315 (1.3% of the total sequences vs 21.2% in COI).

316

317 *Sequencing depth and α -diversity patterns*

318 We metabarcoded a total of 83 samples (36 subsamples from 4 benthic communities in Cíes, 36
319 from 4 communities in Cabrera, 3 samples from a tidal lagoon, 4 additional samples used for
320 studying reproducibility, 2 blanks and 2 negative controls). After the refining procedures, our
321 final dataset for 18S comprised a total of 8,266,952 reads, with an average of 105,987 reads per
322 sample (range: 61,828 – 190,046). For COI, our final dataset included 10,093,453 reads, with an
323 average of 134,580 reads per sample (range: 10,154 – 423,822). One sample from the 18S
324 dataset and four samples from the COI dataset were removed from the analyses due to low
325 number of reads (< 10,000). Controls had a negligible number of reads.

326 The number of total MOTUs detected from all samples by Bayesian clustering was 5,067 for
327 18S, from which 4,130 (81.5%) could be assigned to the level of phylum or lower. These
328 assigned MOTUs accounted for 98.1% of the total 18S reads. As expected, the number of
329 MOTUs yielded by COI from the same samples was higher: 21,452, from which 12,369 (57.6%)
330 could be taxonomically assigned to the level of phylum or lower. The assigned MOTUs
331 accounted for 91.3% of total COI reads. Our final datasets, including the sequences of all
332 MOTUs, their taxonomic assignment and their abundances in each sample, are presented as
333 supplementary material (tables S3 and S4) and are available from the Mendeley data repository
334 (<https://data.mendeley.com/datasets/nm2c97fjng/1>).

335 The different fractions of the sampled communities showed similar patterns of α -diversity for
336 18S and COI after rarefaction (fig. 3). Using either marker, a trend can be observed whereby
337 larger fractions (A and B) had similar values for MOTU richness, whereas the smallest fraction
338 (C) was significantly more diverse than the other two (all Kruskal-Wallis followed by Dunn's
339 tests $p < 0.01$) with the exception of the Mediterranean detritic rhodolith community, where no
340 differences in α -diversity between fractions were detected using COI (Kruskal-Wallis $p = 0.14$).
341 The α -diversity values for the lagoon sediment samples were in the same range than those of
342 samples from fractions C of hard substrate communities. Values of α -diversity measured from
343 COI were roughly three times higher than values obtained from 18S.

344

345 *Taxonomic assignment and database gaps*

346 The number of taxa identified at phylum or lower categories for both markers is shown in fig. 4.
347 A clear trend emerges: the lower the category, the less coincidence between the taxa found with
348 both markers. Thus, at the phylum level, 87.5% of the phyla detected with COI were also
349 recovered with 18S. The corresponding figures were 81.4% for Class, 66.2% for Order, 42.6%
350 for Family, 24.5% for Genus, and 6.5% for species-level taxa. Moreover, 18S detected a higher
351 number of taxa in the different categories, except for species: 803 species were detected with
352 COI vs 615 with 18S.

353 The numbers of MOTUs detected by phylum (fig. 5) showed that both markers, COI and 18S,
354 were able to detect those groups composed of medium- or big-sized organisms, such as major
355 metazoan phyla or macroscopic seaweeds. The detection of groups comprising microscopic

356 organisms was usually more reliable using 18S than COI. For example, 19 metazoan phyla could
357 be detected in our samples using COI, while the 18S assignment detected these same 19 phyla
358 plus the microscopic Kinorhyncha, Loricifera and Gnathostomulida. Due to remarkable gaps in
359 the reference database (as seen in table S2), our assignment procedure for COI was unable to
360 identify any sequence from microscopic groups such as Apusozoa, Choanozoa, Heliozoa,
361 Protalveolata or Rhizaria (including Foraminifera, Cercozoa and Radiozoa), which could be
362 detected by 18S. However, COI was able to detect and distinguish a higher number of MOTUs
363 than 18S for most macroscopic phyla. Moreover, the assignment at the species level was more
364 reliable using COI than 18S. An assignment with an identity percent higher than 97% using COI
365 leads in general to correct species identification; whereas, in many cases, the assignment of 18S
366 by the ecotag algorithm (even at 100% identity) yielded taxa not present in the studied areas.
367 This happens because related species included in the reference database share exactly the same
368 sequence for the 18S fragment used, whereas cases of synonymous sequences for different
369 species are extremely rare using COI. Although errors in taxonomic annotation in the databases
370 can also affect species identification, such errors would be present for both markers. MOTUs
371 with high abundance of reads could be in general identified to the species level using COI,
372 whereas they were often identified to higher taxonomic ranks using 18S (fig. S3). Unassigned
373 MOTUs are those with least abundances, using either marker (fig. S3).

374

375 *Patterns of MOTU abundances*

376 The abundances of reads assigned to major eukaryotic groups at a level of phylum or lower are
377 presented (in percentages) in fig. 6 for COI and 18S (the same information split by replicate
378 samples is presented in fig. S4). The rates of unassigned sequences were, in all cases, higher for
379 COI than for 18S. The unassigned reads were always most abundant in the smallest fraction
380 (fraction C) of each community and were particularly abundant in the lagoon sediment samples
381 (average: 35.7% of COI reads vs 19.7% of 18S reads). Sequences identified as small Metazoa
382 such as Annelida or Arthropoda were also clearly more abundant in the smallest fractions,
383 whereas big macroscopic seaweeds such as Rhodophyceae or Phaeophyceae tended to be
384 dominant in the biggest fractions (A and B). Colonial and modular Metazoa such as Porifera,
385 Cnidaria or Bryozoa were distributed across all fraction sizes. The lagoon sediment samples
386 (unsieved) were enriched in microscopic organisms such as Bacillariophyta (29.4% of COI

387 reads), Oomycetes (2.9% of COI reads) or Ciliophora (6.0% of 18S reads), which were scarce in
388 the sieved samples (averages of 0.75%, 0.37% and 0.20% respectively). Although some
389 differences may be observed between both markers (e.g.: higher abundance of reads of Mollusca
390 and Porifera from 18S and more reads of Arthropoda and Rhodophyta from COI), the overall
391 patterns of read abundances were similar for 18S and COI. The three ecological replicates per
392 community were also similar in composition (fig. S4).

393 The number of MOTUs assigned to the different phyla in each sample are shown (in
394 percentages) in fig. 7 for COI and 18S (the same information split by replicate samples is
395 presented in fig. S5). Compared to the read abundances of fig. 6, a higher dominance of small-
396 sized MOTUs is apparent. The percentages of MOTUs assigned to microeukaryotes were
397 notably higher for 18S than for COI. So, the sum of MOTUs assigned to ciliates, dinoflagellates,
398 Bigyra and other protists accounted for 15.11% of assigned 18S MOTUs, while this sum was just
399 2.27% of the assigned COI MOTUs. Other groups with higher relative richness measured by 18S
400 were annelids (9.56% of 18S MOTUs vs 5.70% of COI MOTUs), nematodes (3.38 vs 0.48%)
401 and flatworms (2.98 vs 0.21%). In contrast, COI detected relatively more MOTUs than 18S for
402 rhodophytes (19.11% COI vs 12.41% 18S), cnidarians (14.9% vs 9.13%), arthropods (15.07% vs
403 11.16%), mollusks (8.93% vs 5.25%), oomycetes (4.11% vs 0.59%) and diatoms (7.13% vs
404 4.38%). All other groups differed in less than 2% of total assigned MOTUs among markers.
405 Again, the three replicates per community showed a similar composition in terms of MOTU
406 richness per phylum (fig. S5).

407

408 *Reproducibility*

409 Weighted UniFrac dissimilarity indices calculated among PCR replicates and among extraction
410 replicates were compared with dissimilarities among ecological replicates. For 18S, the average
411 dissimilarity between PCR-replicates was 0.0003 ± 0.0001 , smaller than between DNA
412 extractions from the same sample (0.0016 ± 0.0016) and two orders of magnitude smaller than
413 between ecological replicates (0.0390 ± 0.0120). For COI, the equivalent values were $0.102 \pm$
414 0.017 , 0.117 ± 0.017 and 0.283 ± 0.016 for PCR-replicates, extraction-replicates and ecological
415 replicates, respectively. Thus, technical replicates yielded significantly more similar results than
416 ecological replicates, indicating the robustness of the protocols. Pie charts representing the read

417 abundances of major groups detected at the different levels of replication are shown in fig. S6,
418 which highlight the differences in the relative abundances of MOTUs among ecological
419 replicates compared to extraction replicates and PCR replicates, with both markers.

420

421 *Ordination patterns of community structure*

422 Non-metric multidimensional scaling (nMDS) plots showing the ordination of the studied
423 communities are shown in fig. S7 for COI and 18S. Similar ordination patterns were recovered
424 from both markers, and the two Bray-Curtis matrices (18S and COI) were highly correlated
425 (mantel test, $r=0.897$, $p<0.001$). Samples from the three fractions of each community grouped
426 together, with overlap of the inertia ellipses in many cases. Samples from both photophilous
427 atlantic communities clustered together, and the same applies to both photophilous
428 mediterranean communities, suggesting the presence of a high proportion of shared MOTUs
429 between these shallow communities. On the other hand, mediterranean and atlantic samples
430 appeared separated, and a gradient from shallower (well-lit photophilic communities) to deeper,
431 sciaphilous communities was apparent. Samples from the shallow lagoon appeared as a tight
432 cluster, well-separated from other benthic communities.

433

434 *Effect of size fractionation in the detectability of MOTUs*

435 Venn diagrams representing the MOTUs detected in the three fractions are presented in fig. 8.
436 There is an important overlap with 18S (73% of MOTUs were detected in the three fractions),
437 while this overlap is substantially reduced with COI (56% of MOTUs). In addition, fraction C of
438 COI has twice as many unique MOTUs as fraction C of 18S, and more than one quarter of COI
439 MOTUs (27%) are found exclusively in the two smaller fractions (B and C).

440 Differences between fractions were more evident in terms of read abundance than
441 presence/absence of MOTUs. The percentages of read abundances belonging to different
442 ecological size-categories recovered from every fraction of the analyzed communities are
443 presented in fig. 9 for COI and 18S. The microorganismal category was recovered mainly in the
444 unsieved samples from the lagoon, which were mostly composed of meiofauna, microorganisms
445 and unassigned reads. Interestingly, most reads of microorganisms detected in fractions A, B and
446 C of hard-bottom communities, belonged to *Symbiodinium sp.* or *Amphidinium sp.*,

447 dinoflagellates which are symbionts of macrofaunal anthozoans. As expected, macroalgae were
448 more abundant in fractions A and B, whereas meiofaunal reads were more abundant in fractions
449 C. Reads of modular metazoans were more evenly distributed among the three fractions.

450

451

452 **Discussion**

453

454

455 The application of metabarcoding techniques to characterize marine hard bottom communities
456 has been hindered by a lack of standardized methods for sample collection and treatment, the
457 scarcity of universal primers capable of amplifying the wide array of taxonomic groups present
458 in these communities and the need of bioinformatic procedures able to cope with the high degree
459 of genetic diversity obtained. We think that the procedures presented here, which include
460 extraction of DNA from separate size fractions, a novel set of highly degenerate primers for COI,
461 capable of amplifying most eukaryotic groups, and improved bioinformatic pipelines for data
462 treatment including new reference databases for Eukarya, allow to overcome many of the
463 challenges related to metabarcoding of structurally complex macroscopic benthic communities.
464 In this work, we tested this approach on the eukaryotic diversity present in eight ecologically
465 diverse littoral benthic communities. These procedures have already proven useful to detect
466 effects of three invasive algae on the small-sized organisms of littoral communities in a different
467 set of samples (Wangensteen et al. in press) and can be applied, with the necessary adjustments,
468 for biodiversity assessment in a wide array of marine, terrestrial or freshwater eukaryotic
469 communities.

470

471 *Sample pre-treatment, the benefits of size fractionation*

472 The partitioned metabarcoding of size fractions filtered through a column of sieves allows
473 characterization of structurally complex communities at different levels, which would be
474 impossible using whole samples, due to the high number of DNA copies from organisms of
475 bigger biomass outnumbering the smaller ones and hampering their detection (Coward et al.
476 2015, Elbrecht et al. 2017). We have shown that the smallest fractions are the most diverse (fig.
477 3) and are enriched in meiofaunal elements, which can be detected because most of the biomass

478 from big-sized organisms is retained within fractions A and B (fig. 9). Even if there is an
479 important qualitative overlap (fig. 8), many MOTUs appear in some abundance only in fraction
480 C. Thus, without fraction C, not only the exclusive MOTUs disappear, but 280 MOTUs of 18S
481 (5.5% of the total) and 3,317 MOTUs of COI (15.4%) were left with less than 5 reads. These
482 MOTUs would have been removed during the minimal filtering clean-up without the
483 contribution of reads from the smallest fraction.

484 An additional advantage of this procedure is the removal of a significant fraction of
485 microorganisms (prokaryotes and the smallest microeukaryotes), together with most of the extra-
486 organismal DNA in the form of small remains, cell debris, or extracellular DNA (Creer et al.
487 2016), which are not retained in the last sieve (63 μ m). Microeukaryotes are known to be
488 genetically diverse and under-represented in genetic databases, which introduces problems
489 during bioinformatic analyses, particularly for clustering and taxonomic assignment algorithms.
490 They are better removed from the samples by sieving whenever they are not the main study
491 target. The improved results in the assignment can be appreciated by the higher rate of
492 unassigned reads from the tidal lagoon (unsieved) compared to sieved samples from littoral
493 communities (figs. 6 and 9). Moreover, most MOTUs with high read abundances could be
494 assigned to the species level using COI (fig. S3), while unassigned MOTUs were typically the
495 least abundant, suggesting again the reference database bias towards big and abundant species.
496 Therefore, in studies mainly aimed at characterizing macro- and meio-benthic components, some
497 physical filtering step is advisable during sample pre-treatment. Size-fractionation has been used
498 to separate relevant compartments in metabarcoding studies of planktonic organisms (e.g.,
499 Logares et al. 2014, Massana et al. 2015, Liu et al. 2017) and in some studies of sedimentary
500 bottoms (e.g. Chariton et al. 2010, Coward et al. 2015, Aylagas et al. 2016). However, this point
501 had not been addressed for hard-bottom benthic communities, where size-differences encompass
502 many orders of magnitude. The closest reference is the study of artificial settlement surfaces
503 (ARMS, Leray & Knowlton 2015, Ransome et al. 2017) where organisms were separated into
504 sessile biota (processed in bulk) and three size-classes of motile organisms, being the smaller
505 fractions the most diverse. Size-fractionation should be mandatory for adequate recovery of
506 biodiversity in hard substrate benthic communities.

507

508 *Choice of a proper metabarcoding marker, COI vs 18S*

509 The amplification of COI resulted in more MOTUs than 18S (by a factor of 4) and more
510 resolving power at the species level (803 vs 615 species-level assignments), at the cost of a
511 higher proportion of unassigned MOTUs (overall 42.4% and 18.5%, respectively), a result
512 consistent with previous findings (e.g., zooplankton Clarke et al. 2017). The use of COI as a
513 metabarcoding marker has been criticized in the past, arguing that high rates of sequence
514 variability impair the design of truly universal primers and hamper the bioinformatic analyses
515 (Deagle et al. 2014), but attempts have been made recently to incorporate COI data in
516 metabarcoding studies (e.g., Leray and Knowlton 2015, Berry et al 2015, Aylagas et al. 2016,
517 Elbrecht & Leese, 2017). . However, COI presents two major advantages compared to other
518 possible markers. First, the steadily growing international effort to develop a public DNA
519 barcoding database with curated taxonomy, which vastly facilitates taxonomic assignment. The
520 BOLD database (Hebert et al. 2003; Ratnasingham & Hebert 2007), based mainly in COI
521 barcoding, currently includes over 4 million sequences belonging to more than 500,000 different
522 species, curated and identified by expert taxonomists. It is highly unlikely that any comparable
523 effort might be undertaken for any other marker in the next future. Second, the high mutation
524 rate of COI practically ensures unequivocal identification at the species level, whereas the highly
525 conserved sequence of 18S makes it usually impossible to distinguish at the genus or family
526 levels, or even at higher ranks (Tang et al. 2012). Species-level resolution is crucial for
527 calculating ecological indices or detecting non-native species (Comtet et al. 2015, Aylagas et al.
528 2016).

529 Overall, then, we favour the use of COI amplicons for characterizing complex marine
530 communities. The use of 18S can be recommended only when the information at the species
531 level is not crucial. For example, to assess overall impacts related to human activities such as
532 fisheries, aquaculture or mining facilities. In this case, the impact may be expected to affect
533 abundances and composition at higher taxonomical levels. Studying these impacts using 18S
534 may benefit from the less computationally demanding and faster bioinformatics processing of
535 18S data than that of COI data.

536 Our universal Leray-XT primer set for COI features high values of *in silico* coverage (fig. 2) and
537 was able to successfully amplify a wide range of eukaryotic organisms belonging to 19 phyla of
538 Metazoa and all major marine lineages of Eukaryota (fig. 5). The undetectability of some lesser
539 groups in this study is more related to the incompleteness of reference databases (table S2),

540 rather than to PCR failure of our COI primer set. We recommend the use of this universal primer
541 set (mlCOIintF-XT and jgHCO2198) for COI metabarcoding analyses of marine samples or
542 other environmental or community DNA projects, especially when a wide taxonomic range of
543 eukaryotes is expected and species-level resolution is necessary. We note, however, that these
544 primers have limited ability for detecting some groups (e.g., Viridiplantae and Ciliophora, fig. 2,
545 table S2). Thus, specific primers or a different marker should be used if these taxonomic groups
546 constitute the main study target.

547 It is remarkable that ordination analyses of our data yielded robust and comparable results,
548 disregarding the marker chosen (fig. S7). The two distance matrices were highly correlated,
549 indicating that the same general ecological information is retrieved with both markers. This
550 implies that robust and objective methods for impact studies or comparisons among communities
551 may be designed and implemented with different markers.

552

553 *Estimates of α -diversity: a comparison with morphological studies.*

554 The ultimate aim of metabarcoding is to objectively determine which species are present in a
555 given environmental sample. The values obtained for eukaryotic richness in this study are
556 astoundingly large. Using 18S, 4,203 different MOTUs were detected in the four mediterranean
557 communities combined, and 3,914 MOTUs in their atlantic counterparts. The respective values
558 for COI MOTU richness are 14,092 for Cabrera and 13,708 for Cies. These values are
559 comparable to 17,000, which is the total number of morphological species described for the
560 whole Mediterranean Sea (Coll et al. 2010). Rarefaction to just 10,000 reads per sample (fig. 3)
561 yielded values for MOTU richness of roughly 200-600 MOTUs per replicate using 18S or 500-
562 1500 MOTUs per replicate using COI. However, an adequate benchmarking of these values
563 against richness detected with traditional (morphology-based) techniques for this kind of
564 communities is still lacking. For sediment macroinvertebrates, Aylagas et al. (2016) showed that
565 the Leray fragment generated over 50% of matches (depending on the protocol and lab
566 conditions tested) between morphologically and molecularly inferred taxonomic composition.
567 Biotic indices based on both sources were also well correlated. We cannot perform such a direct
568 comparison with our samples as morphological information is not available. However, we can
569 draw upon published studies of the same communities analyzed to gain an idea of the relative

570 performance of metabarcoding for characterizing biodiversity in hard substratum communities.

571 Traditional methods for community characterization in these communities rely on randomly
572 allocating standardized sampling units (usually quadrats of 20x20 or 25x25 cm) and either
573 collecting the biota through scraping, performing in situ visual censuses, or analyzing
574 photographs. A comparison of the three methods was made precisely on the Cabrera Archipelago
575 (Sant et al. 2017), highlighting relative differences in information and cost/benefits among
576 methods. However, even the best performing method (scraping) identified a total of 262 species,
577 an order of magnitude lower than we obtained in Cabrera from just the coarser fraction (A):
578 3,085 MOTUs with 18S and 9,333 with COI.

579 Other studies have analyzed species richness of the macrofauna and macroflora in both National
580 Parks or geographically close areas in communities identical or similar to the ones studied here.
581 In addition, a monograph is available on the taxonomy of benthic groups in Cabrera Archipelago
582 (Alcover et al. 1993). In table 1 we have collated the information from these works and
583 compared richness values with the ones obtained in our study of the corresponding communities.
584 Metabarcoding largely outperforms morphological inventories, detecting on average 3.16 and
585 8.88 times more MOTUs (18S and COI, respectively) than reported in exhaustive morphological
586 studies. Only in the case of Chlorophyta with both markers, Echinodermata of Cabrera with both
587 markers, Mollusca with 18S and Phaeophyceae in the detritic of Cabrera with 18S did we detect
588 a lower number of MOTUs than morphospecies reported. We must keep in mind that published
589 results are often compilations of several works, while we have results for only a handful of
590 samples taken at a single time point. The dominant genera and species mentioned in quantitative
591 studies are in agreement with the results obtained with metabarcoding (tables S3 and S4).

592 Our results show that genetic estimates for diversity (especially those obtained from COI
593 metabarcoding) largely exceed the results from morphological assessments, in agreement with
594 other metabarcoding studies which reported unexpectedly higher genetic than morphological
595 diversity estimates in comparable samples (Cewart et al. 2015), suggesting the existence of a
596 large number of yet undescribed marine taxonomic lineages. Overall, then, metabarcoding seems
597 well suited for biodiversity detection in hard bottoms, with the added advantage that it can target
598 not just macro-organisms as most previous morphological studies did, but also meio- and micro-
599 organisms. A more precise benchmarking, analyzing the same samples with both methods,
600 remains to be performed in future studies.

601

602 *Taxonomic assignment. Current gaps in databases*

603 We did not add any new sequences to build our custom reference database. Instead, we
604 deliberately used only sequences already available from public repositories in order to assess the
605 completeness of current barcoding databases for marine taxa. Release r117 of the EMBL
606 repository was searched using *in silico* ecoPCR (Ficetola et al. 2010) with our metabarcoding
607 primer sets in order to obtain the reference sequences for our 18S and COI reference databases.
608 Our COI reference database was then enriched by adding sequences available from the BOLD
609 database (Ratnasingham & Hebert 2007). The rates of unassigned sequences in our results
610 suggest that important gaps still exist for both markers in the genetic repositories, which would
611 prevent the detailed identification of many marine organisms, in agreement with the concerns
612 expressed by other authors (Leray & Knowlton 2016). If a fine taxonomic identification of the
613 obtained sequences is desired for a given metabarcoding project, it is advisable not to rely
614 exclusively in the public repositories and obtain custom databases, including the generation of
615 sequences for known local species absent from the repositories. For many ecological
616 applications, however, it suffices that a particular MOTU is defined, its patterns of distribution
617 and abundance assessed, and changes over time monitored, even if a scientific name for that
618 MOTU is yet unavailable (Cordier et al. 2017). Moreover, the sequences of all MOTUs
619 (identified or not) detected by metabarcoding will remain in public repositories, so that
620 unidentified MOTUs might well be assigned a name in the future, as databases improve. The
621 same can hardly be said of morphological studies, where many taxa cannot be identified to
622 species level either, and are left inventoried under general names (e.g., “Nematoda spp or spX”)
623 without descriptions. Therefore, unlike metabarcoding datasets, currently used morphological
624 inventories contain a great deal of untraceable information that can never be used by other
625 researchers at any other place or time.

626 Database gaps affect the metazoan groups differentially. For example, despite being abundant
627 and diverse in benthic ecosystems, most bryozoans and cnidarians could be rarely assigned using
628 COI below the class or order level, whereas species of echinoderms, decapods or vertebrates
629 were usually successfully identified. A trend can also be seen of smaller sized groups, such as
630 Nematoda, Rotifera or benthic Copepoda, being left out of the databases, whereas bigger sized or
631 commercially important animals such as fish or decapods are well represented. There is no doubt

632 that taxonomic assignment of COI metabarcoding data will be more accurate and detailed in the
633 future, as reference databases are populated by international barcoding initiatives, such as the
634 Census of Marine Life (<http://www.coml.org>) or the Marine Barcode of Life (MarBOL,
635 <http://www.marinebarcoding.org>). We strongly advocate for the continued public support and
636 funding of such collaborative DNA-barcoding projects as a necessary tool towards the
637 implementation of reliable and objective metabarcoding techniques for environmental
638 assessment.

639 In this article, we showed how complex communities with organisms of a wide range of sizes
640 can be tractable with an adapted community-DNA metabarcoding approach, even without the
641 availability of taxonomic expertise, thus expanding the range of applications of this technique to
642 ecosystems of enormous ecological and economic importance. At the same time, we have
643 generated the first metabarcoding inventories for natural hard substrate communities, using
644 Marine Protected Areas as our sampling settings, thus providing baseline information for future
645 conservation-oriented research.

646

647

648 **Conclusions**

649

650 In this work we develop and apply a metabarcoding protocol for complex natural marine hard-
651 bottom communities. Size fractionation is mandatory to adequately capture information for
652 organisms of a range of sizes spanning several orders of magnitude. We assayed a novel primer
653 set for the amplification of the “Leray fragment” of COI, introducing more degenerate positions
654 for increased universality, as shown in *in silico* tests. Results show that COI recovers four times
655 more diversity (in MOTU richness) than sequences of the ribosomal 18S molecule (v7 region).
656 Reference databases are generated from publicly available sequences, showing that significant
657 gaps still prevent complete taxonomic assignment of the sequences. Notwithstanding, assigned
658 (at the phylum level) MOTUs represented >90% of reads for both markers. Our results show that
659 marine metabarcoding, currently applied mostly to plankton or sediments, can be adapted to
660 characterize the bewildering diversity of marine benthic communities dominated by macroscopic
661 seaweeds and colonial or modular sessile metazoans. We used as a case study representative

662 sublittoral communities National Parks, thus generating baseline inventories for future
663 biomonitoring of these communities with conservation and management implications.

664

665 **Acknowledgements**

666

667 We are indebted to the staff of the Atlantic Islands of Galicia and Cabrera Archipelago National
668 Parks for sampling permits and invaluable logistic help. Thanks also to Xavier Roijals for his
669 skilful bioinformatic assistance in the CEAB computing cluster facilities. We dedicate this work
670 to Alex Macía, owner of the Cies Diving club, who provided logistics in the Atlantic Islands, and
671 who recently died while following his passion for diving.

672

673

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675

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848

849 **Data Accessibility**

850

851 The resulting datasets for COI and 18S, including sequences, taxonomic assignment and
852 abundances for all MOTUs in every sample, have been deposited in the Mendeley data
853 repository (<https://data.mendeley.com/datasets/nm2c97fjng/1>). Databases of reference sequences
854 to be used for ecotag taxonomic assignment of COI and 18S are deposited in Github
855 (<http://github.com/metabarpark/Reference-databases>). R scripts used as part of the analysis
856 pipeline are also deposited in Github (http://github.com/metabarpark/R_scripts_metabarpark).

Table 1 (on next page)

MOTU richness and morphospecies richness.

Comparison of MOTU richness values obtained in the present work with morphospecies diversity found with morphological methods in previous studies on the same or similar and geographically close communities.

1

Studied area	Community	Group	Morphospecies richness	MOTU richness 18S	MOTU richness COI	References
Ria de Vigo	Cíes detritic	Rhodophyta Phaeophyceae Chlorophyta	123 19 18	303 35 15	842 156 5	Peña & Bárbara 2008
NW Portugal	Cíes C. <i>tamariscifolia</i>	Rhodophyta Phaeophyceae Chlorophyta Polychaeta Crustacea Mollusca Echinodermata	30 12 4 45 35 9 4	183 50 10 210 270 143 23	470 178 4 258 879 604 17	Marques et al. 1982
Balearic Islands	Cabrera precoralligen	Rhodophyta Phaeophyceae Chlorophyta	85 12 5	235 41 11	747 107 3	Ballesteros 1994
Mediterranean Spain	Cabrera detritic	Rhodophyta Phaeophyceae Chlorophyta	197 44 31	268 34 12	623 88 4	Peña & Bárbara 2008
Cabrera archipelago	Cabrera all communities	Macroalgae Porifera Cnidaria Bryozoa Decapoda Mollusca Ascidiacea Echinodermata	277 98 85 145 69 169 41 53	458 250 310 290 47 138 50 25	1676 726 1336 696 32 375 138 28	Ballesteros 1993 Uriz 1993 Gili et al. 1993 Zabala 1993 Corbera et al. 1993 Altaba 1993 Turon 1993 Munar 1993

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3

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Figure 1(on next page)

Natural benthic communities sampled in this study.

(a) photophilous community with *Cystoseira tamariscifolia*, (b) photophilous community with *Cystoseira nodicaulis*, (c) sciaphilous with *Saccorhiza polyschides*, (d) Atlantic detritic bottoms (maërl), (e) photophilous community with the invasive seaweed *Lophocladia lallemandii*, (f) photophilous Mediterranean seaweeds, (g) sciaphilous precoralligenous outcrops, (h) Mediterranean detritic bottoms (maërl). (a)-(d) from Galician Atlantic Islands National Park, (e)-(f) from Cabrera Archipelago National Park. In (a) the 25-cm quadrat sampling unit is shown.

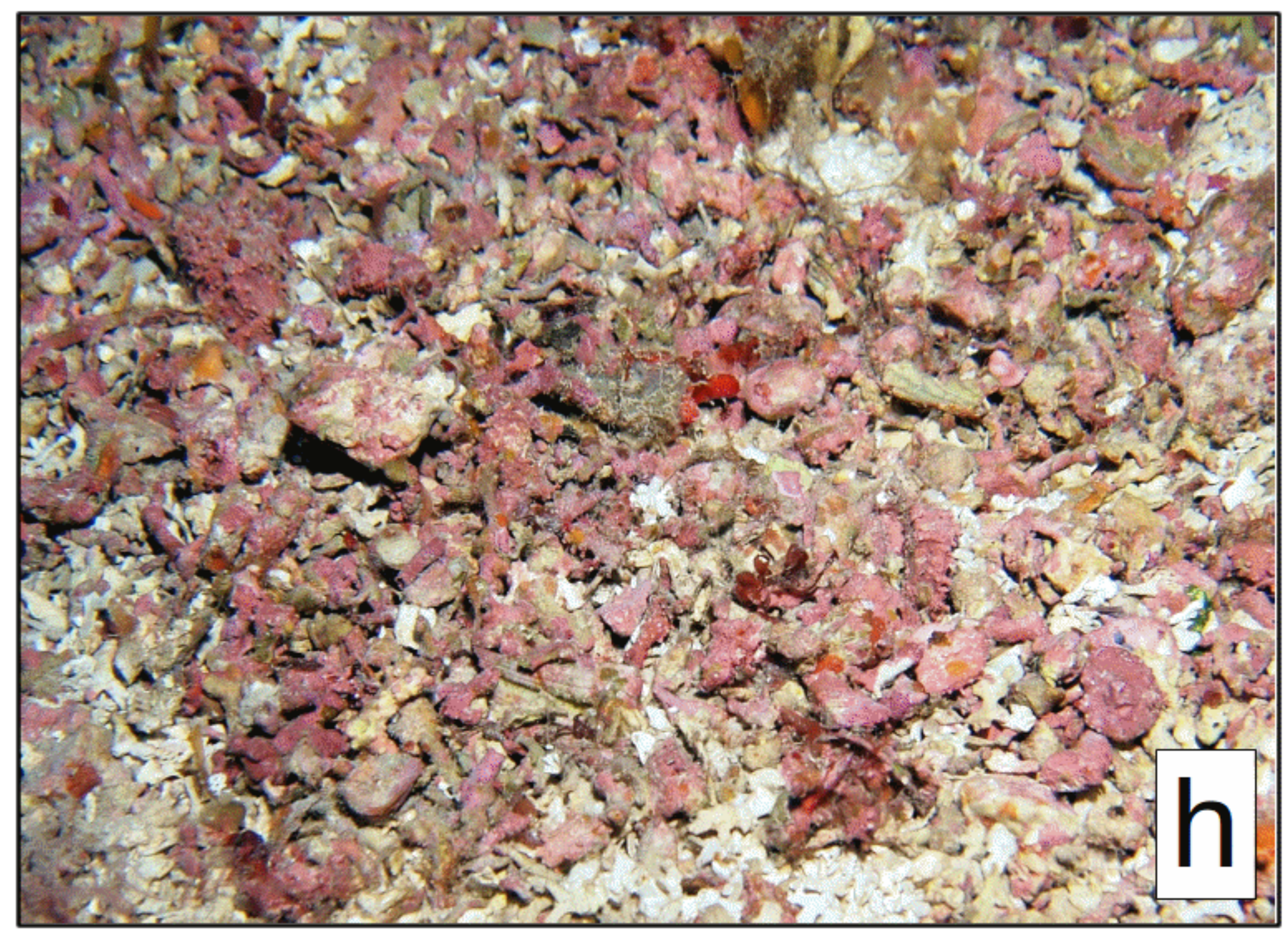
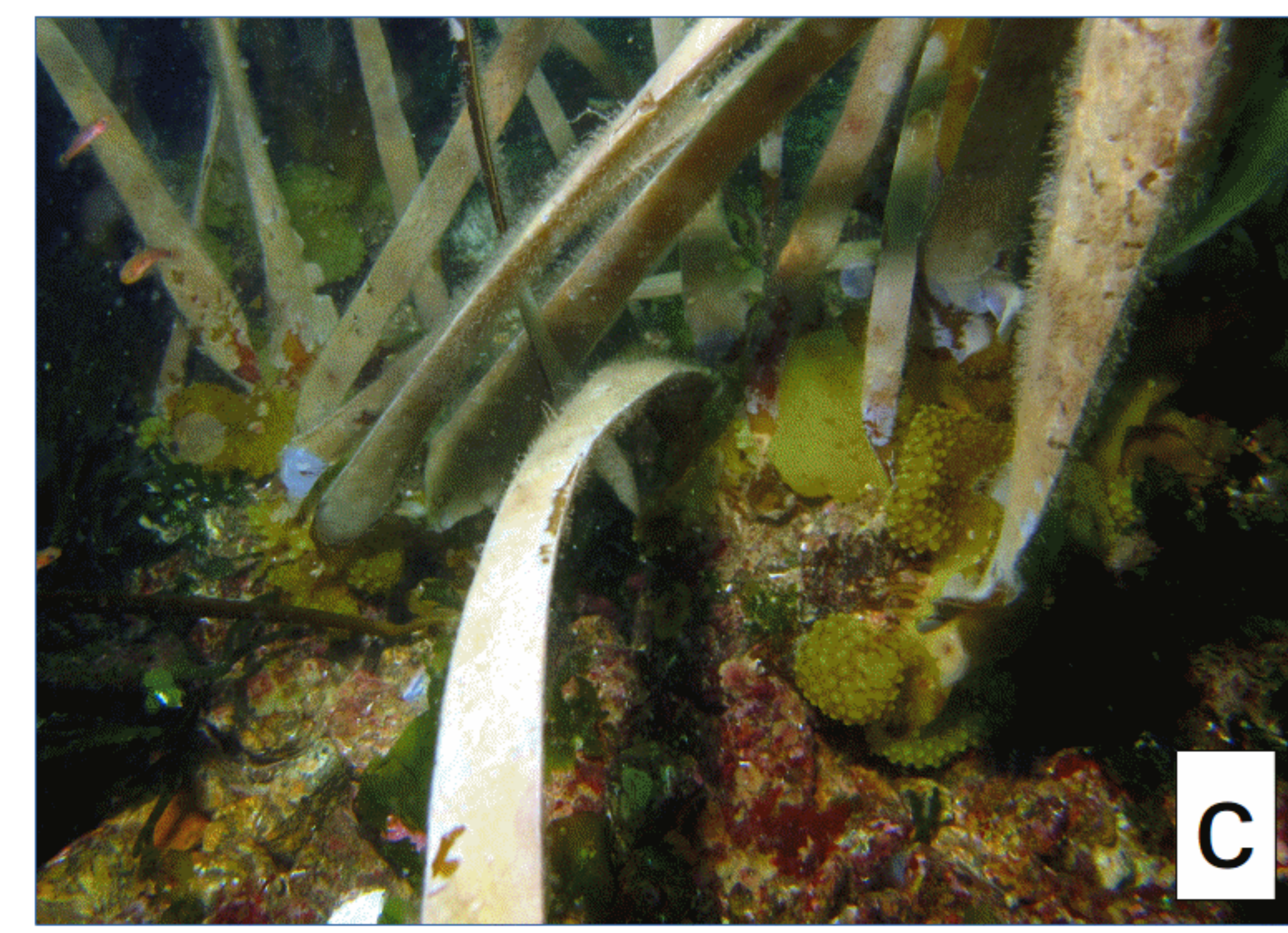
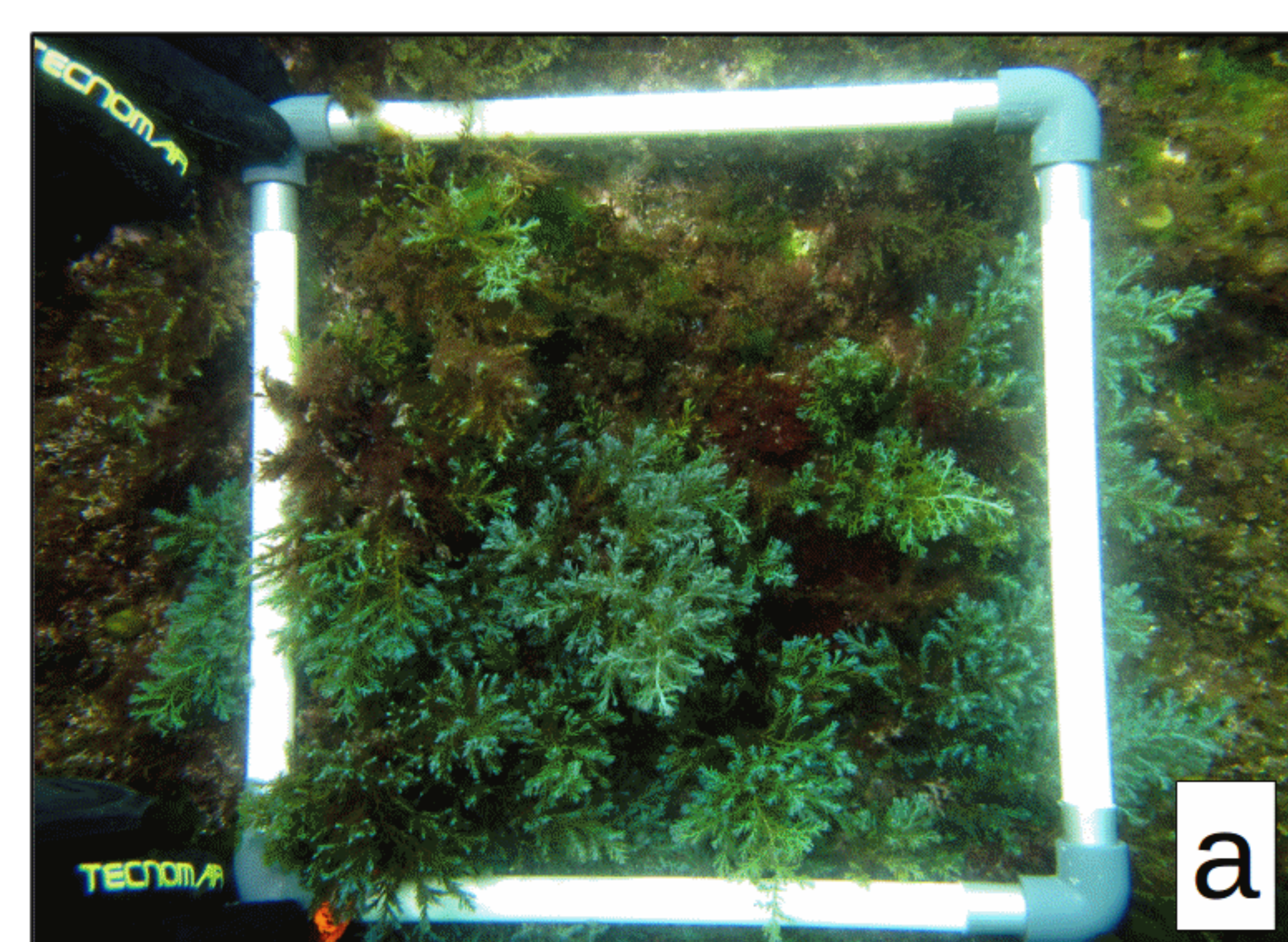


Figure 2

Percentages of *in silico* taxonomic coverage per species using the new Leray-XT primer set for COI.

Figures are compared to the original Leray primer set (Leray et al. 2013), for the main eukaryotic groups (above) and the metazoan phyla (below).

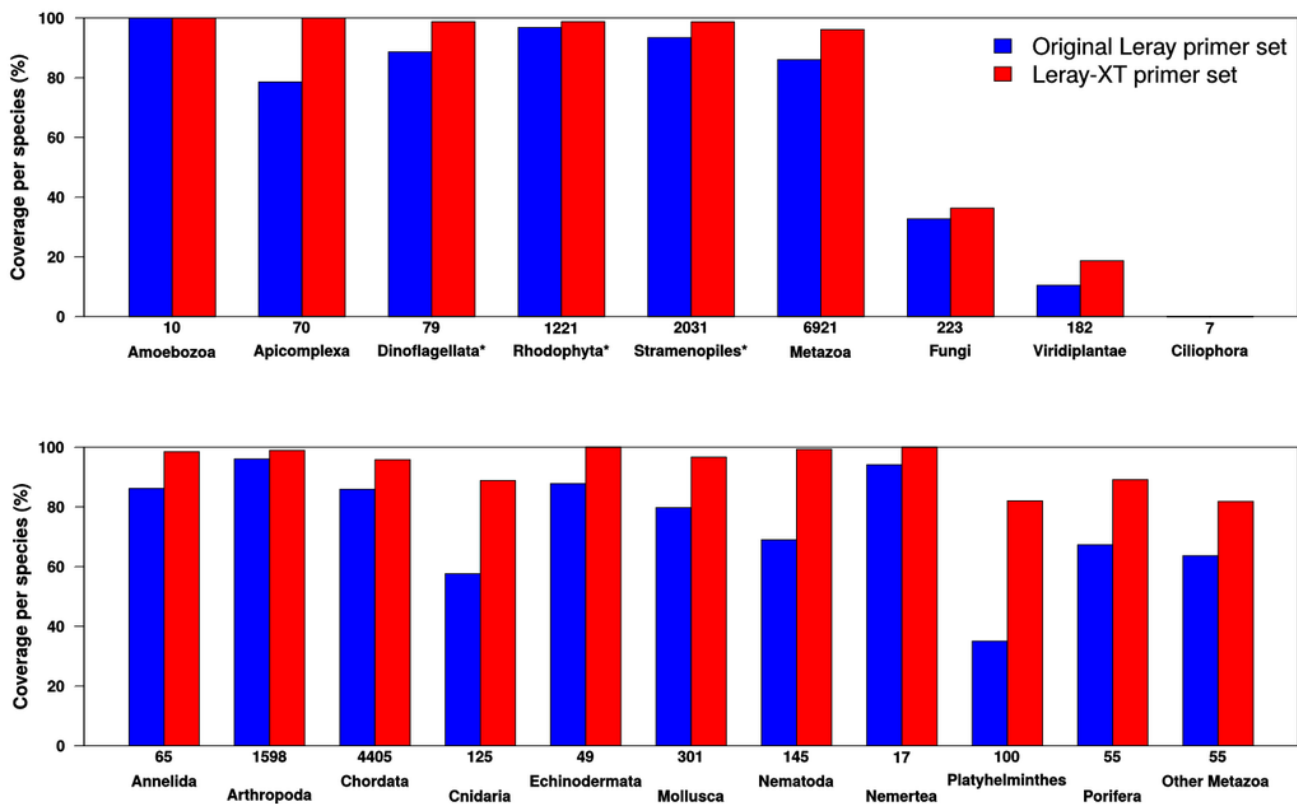


Figure 3(on next page)

Violin plots showing patterns of α -diversity.

Graphs of three different size fractions (A, B and C) in eight different hard substrate marine communities and a set of tidal lagoon sediment samples for comparison purposes. Results obtained by rarefaction analysis to 10,000 reads per sample with 500 replicates. a: MOTU richness obtained from COI. b: MOTU richness obtained from 18S.

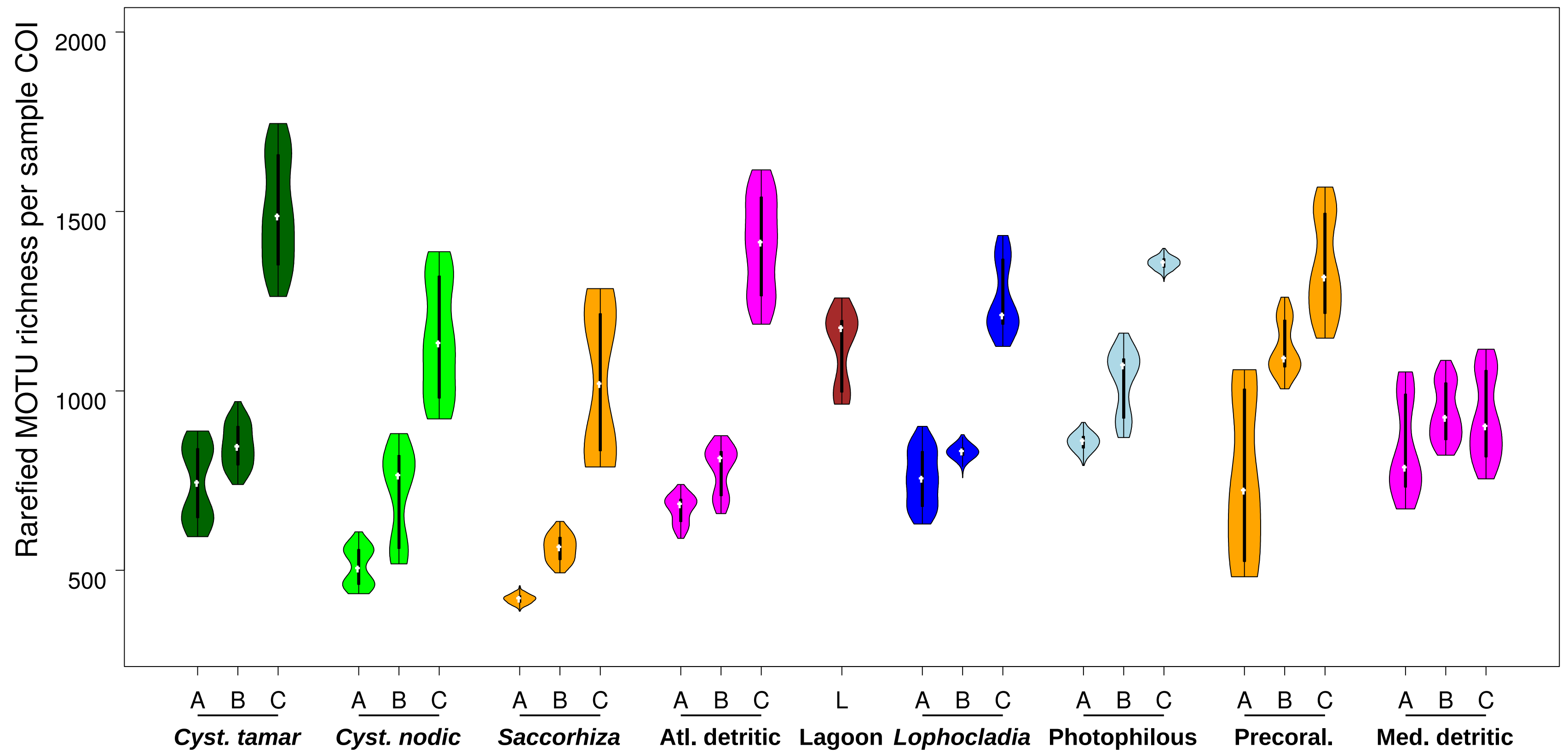
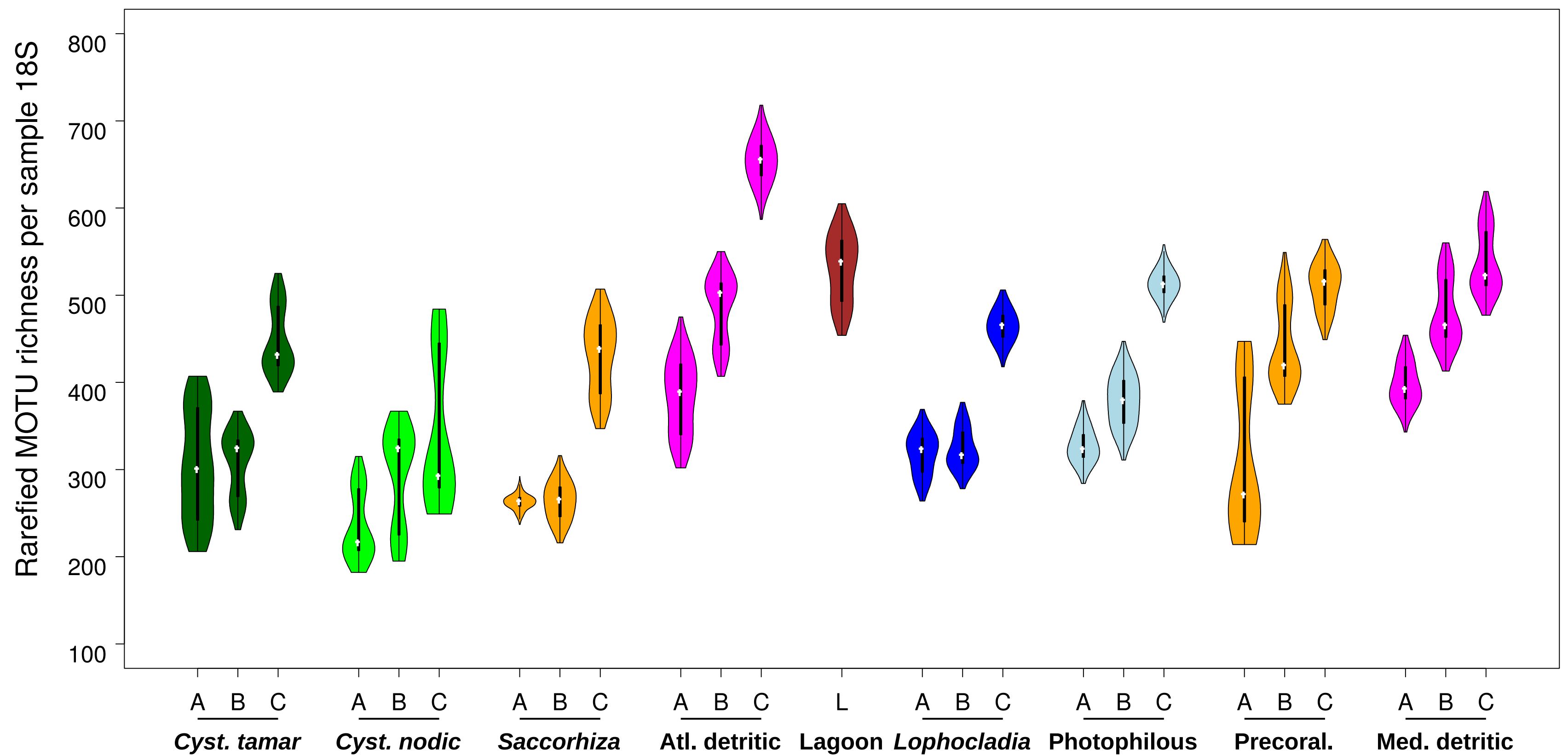
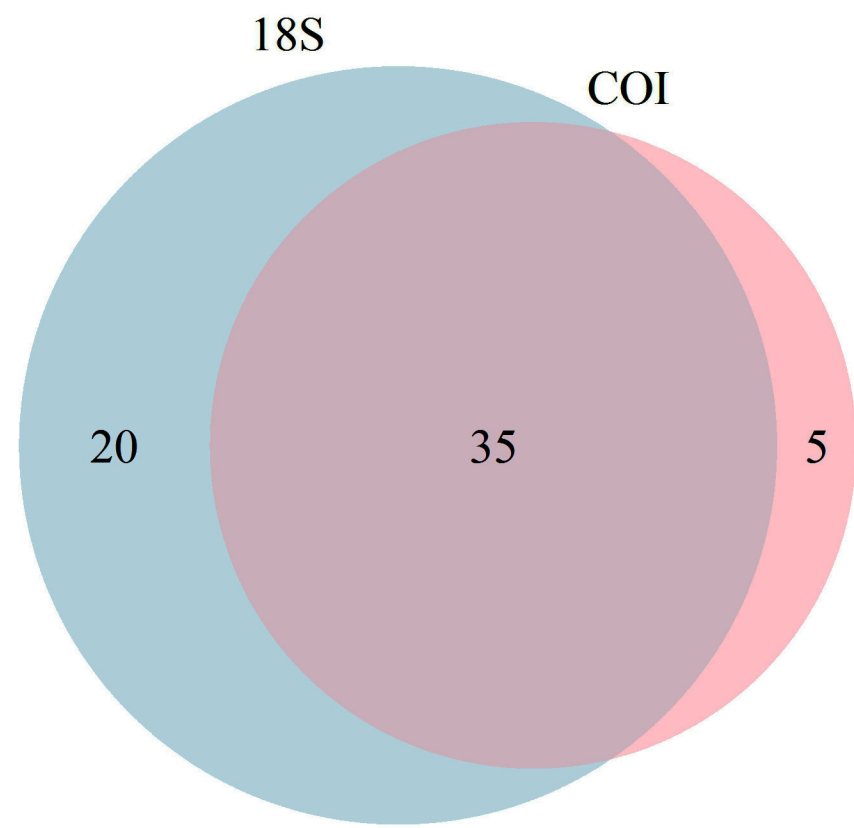
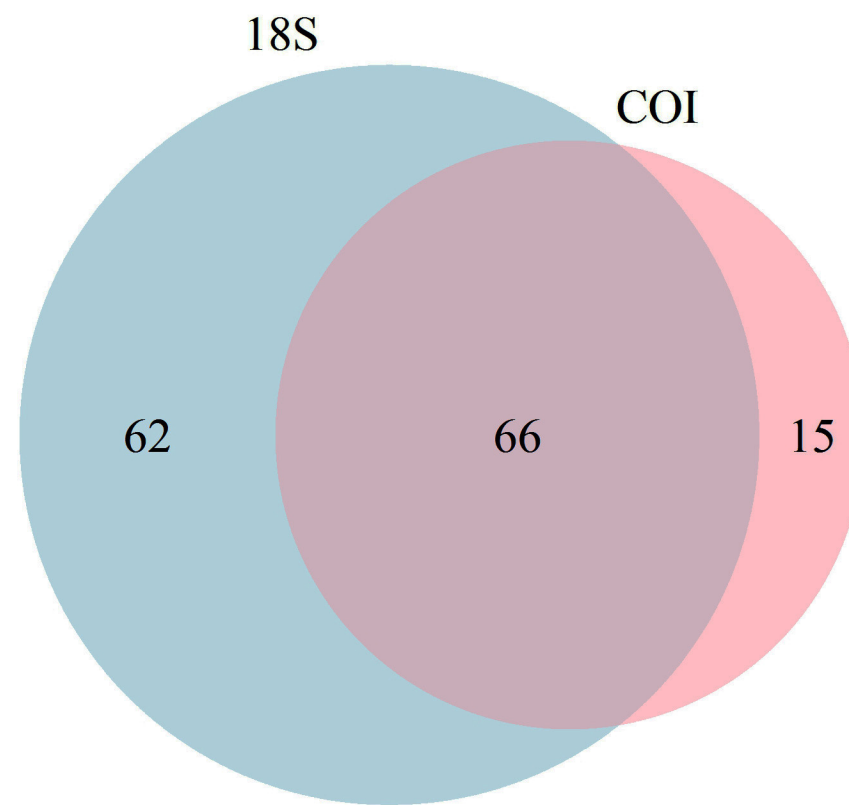
a**b**

Figure 4(on next page)

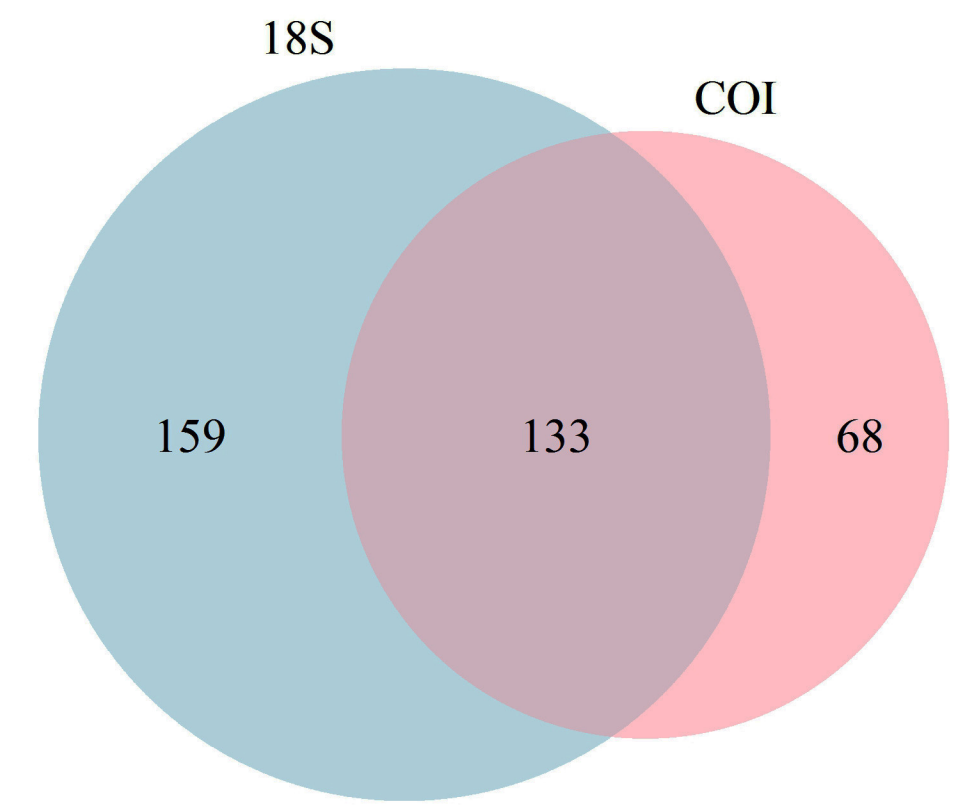
Venn diagrams comparing the number of different taxa recovered using COI (red) or 18S (blue) from all studied communities, for different taxonomic ranks .



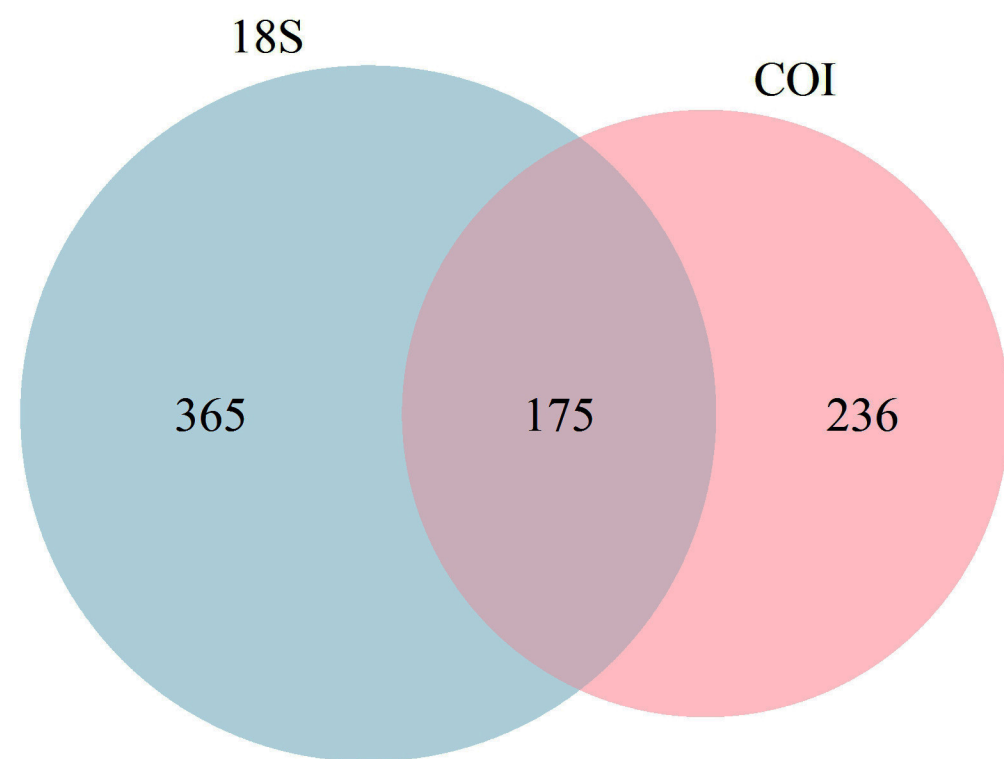
Phylum



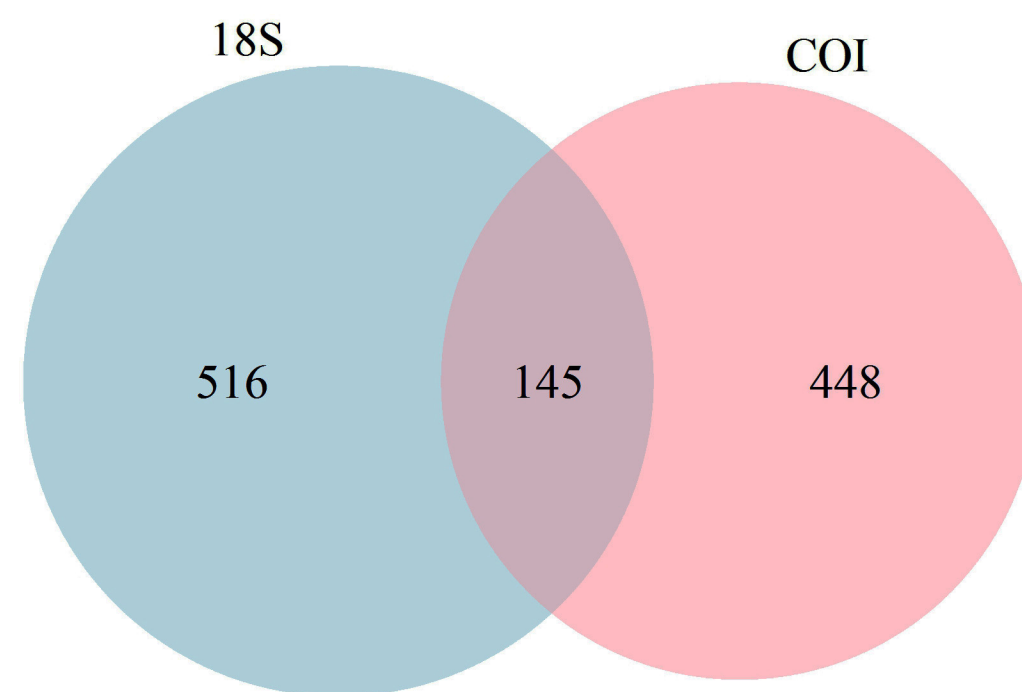
Class



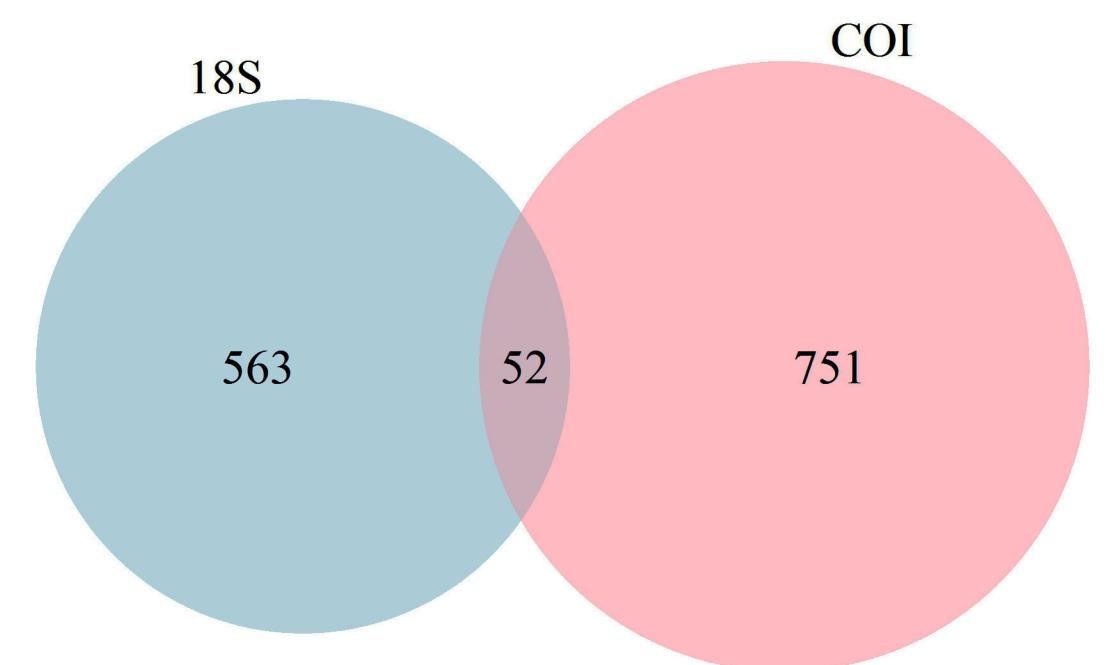
Order



Family



Genus

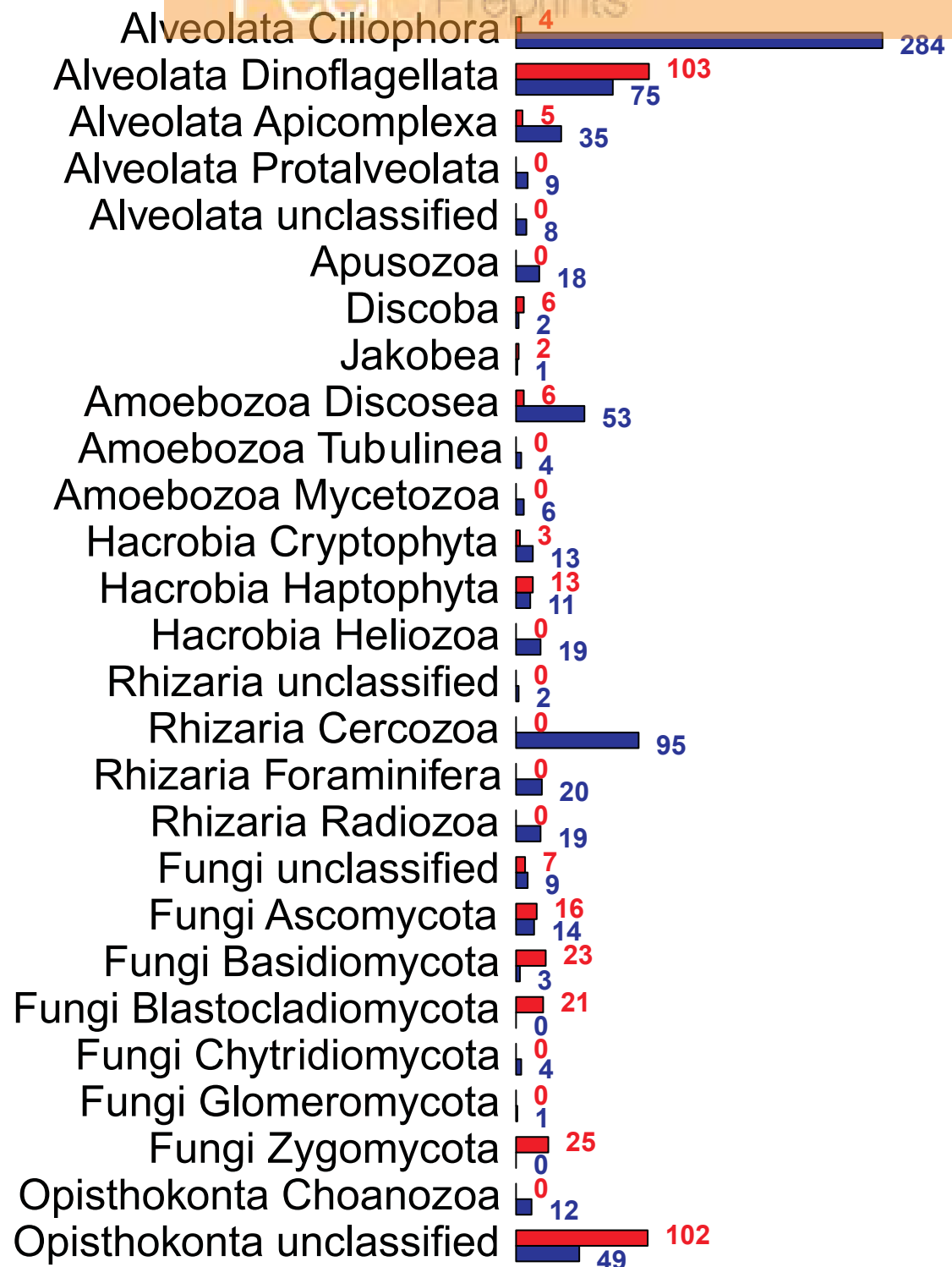


species

Figure 5 (on next page)

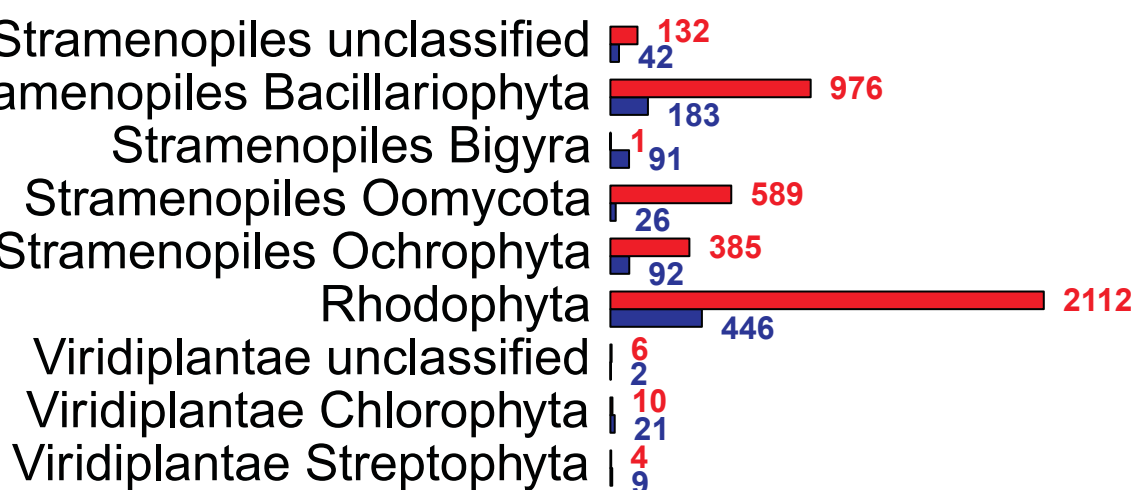
Number of MOTUs detected for every phylum in the studied communities using COI (red) or 18S (blue) metabarcoding.

Note the different scales used for small-sized groups (protists, fungi and small metazoans) and for dominant groups in benthic communities (metazoans and seaweeds).

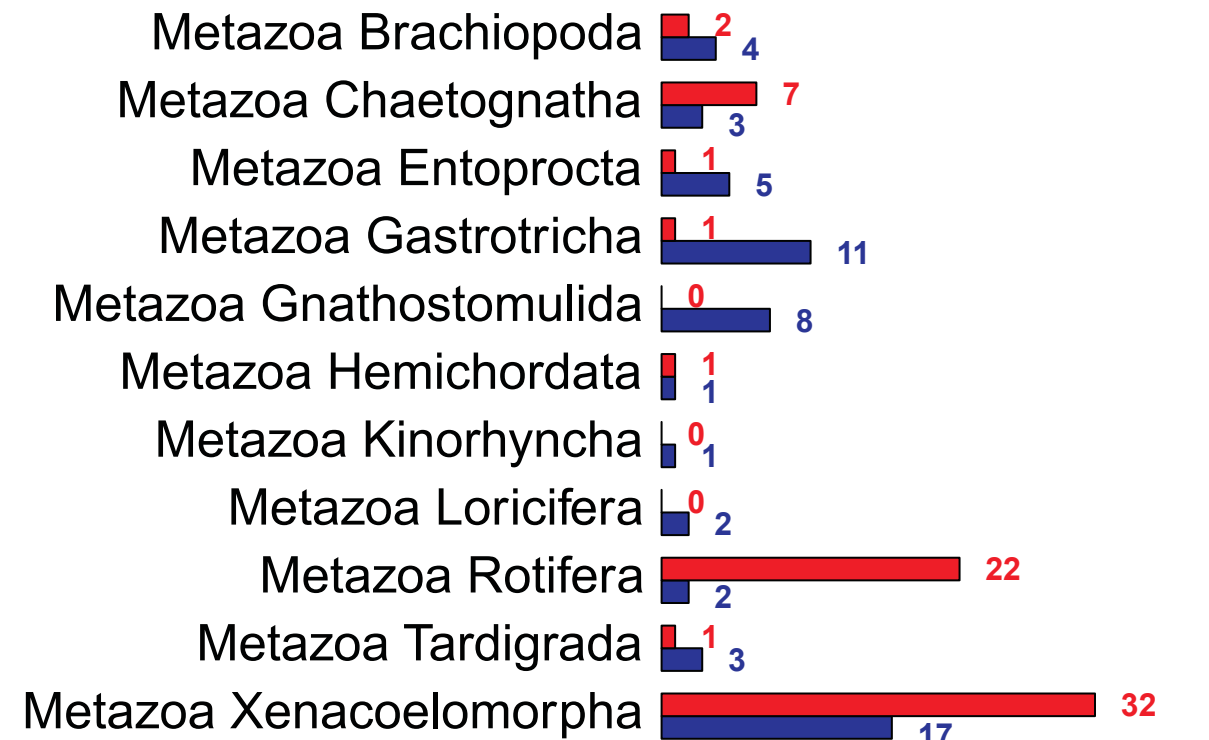


0 50 100 150 200 250

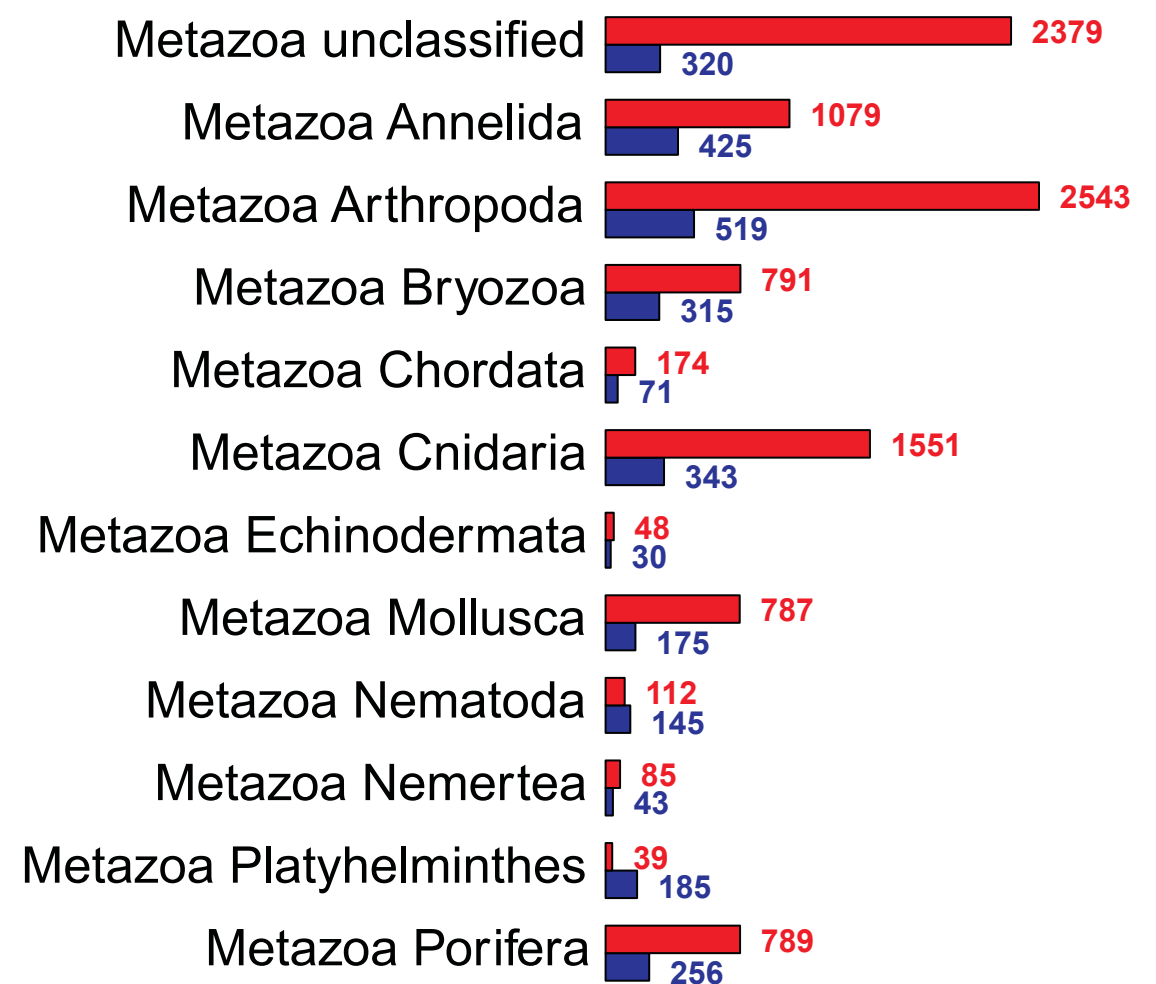
Number of MOTUs detected



0 500 1000 1500 2000



0 5 10 15 20 25 30



0 500 1000 1500 2000 2500

Number of MOTUs detected

Figure 6

Patterns of abundance of metabarcoding reads per community and fraction size.

Results obtained using COI (a) or 18S (b) in eight different marine littoral communities from NE Atlantic (left) and W Mediterranean (right), and a set of lagoon sediment samples. All replicates from the same community and fraction size have been pooled. See supplementary fig. S4 for the same figure, split by replicates.

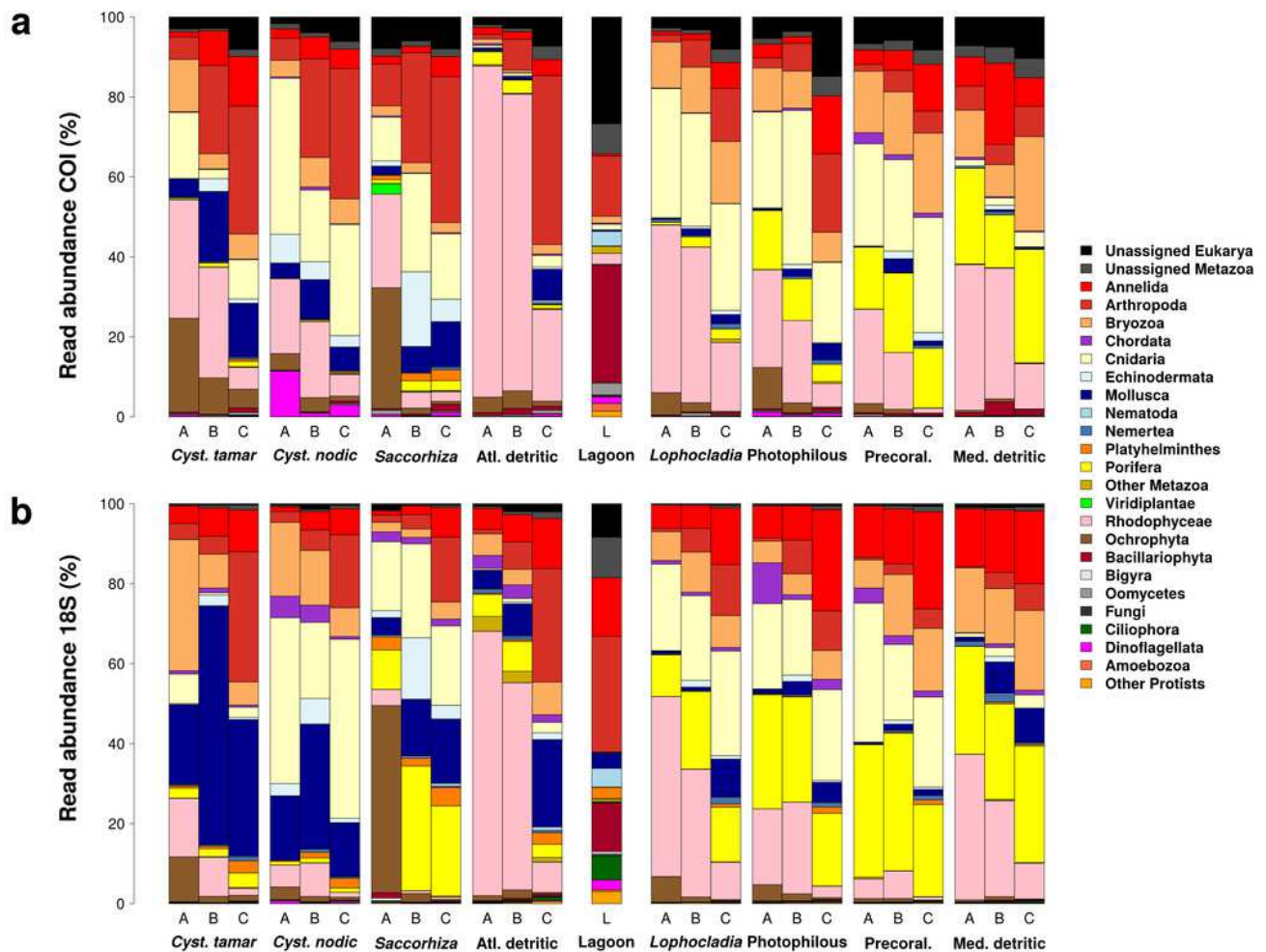


Figure 7

Patterns of relative MOTU richness using COI (a) or 18S (b) in the same communities of fig.6. See supplementary fig. S5 for the same figure, split by replicates.

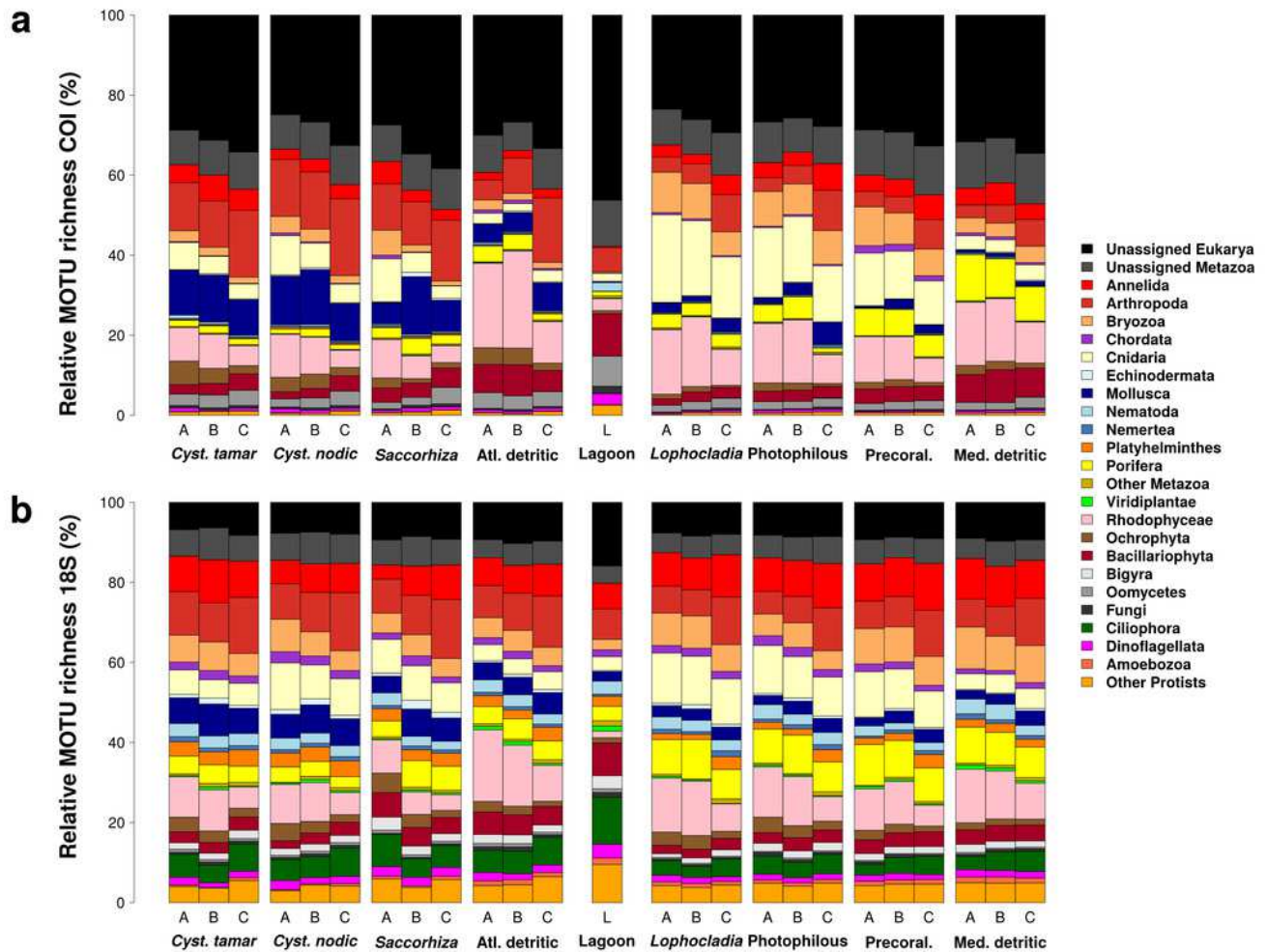


Figure 8(on next page)

Venn diagram showing the number of MOTUs detected in each size fraction (A, B or C) for 18S (left) and COI (right) in all communities. Numbers are percentages of total MOTUs.

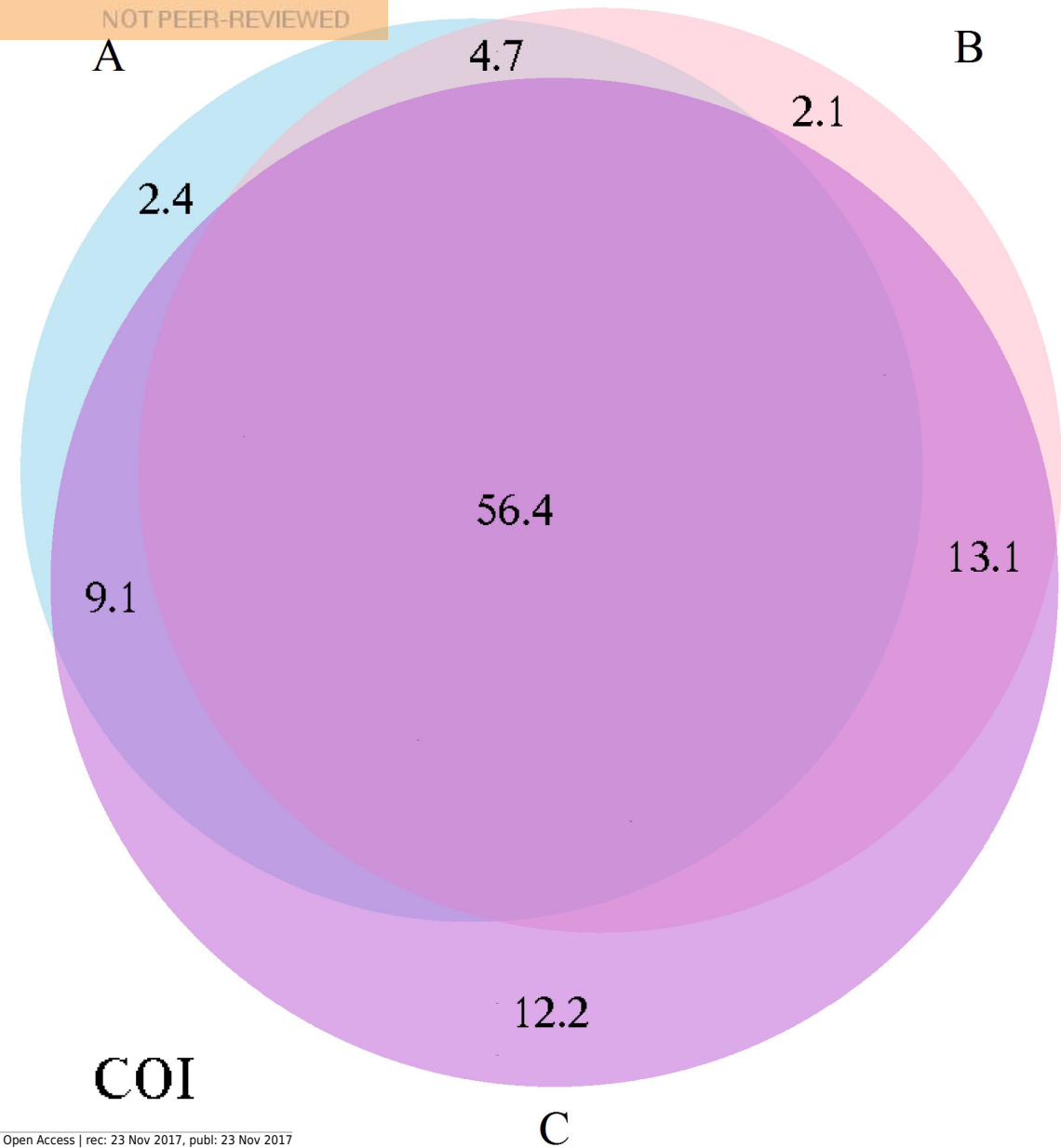
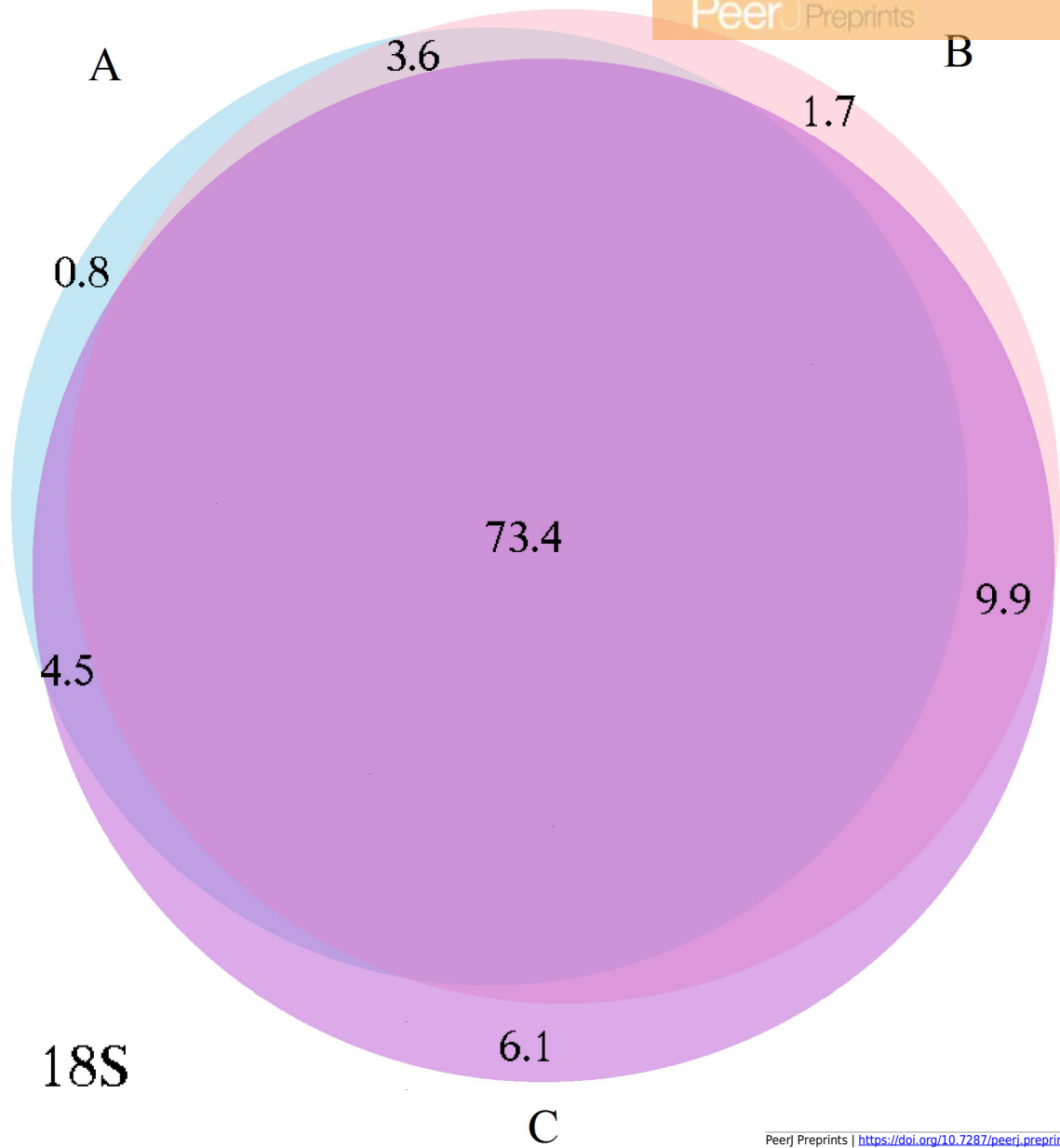
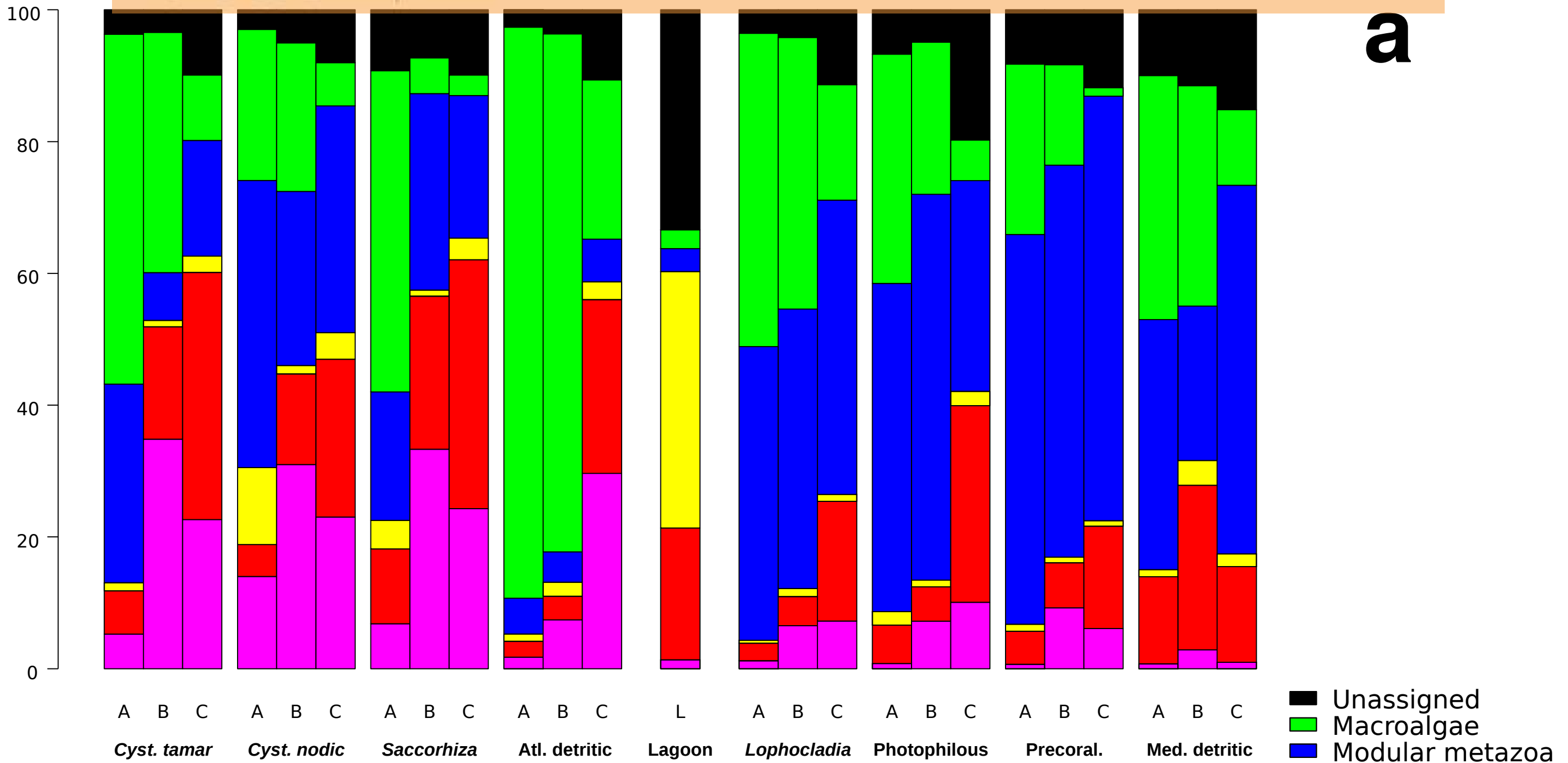


Figure 9 (on next page)

Effect of size fractionation in the recovery of different ecological categories by metabarcoding.

Results using COI (a) and 18S (b) on eight different littoral communities from national parks and sediment samples from a tidal lagoon.

a



b

